Land-based Test according to IMO Guideline G8 of the Hamworthy Water Systems^{Ltd} Aquarius^{TM}-UV Ballast Water Treatment System (2011-2012)

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NIOZ Royal Netherlands Institute for Sea Research

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Signed on Texel, The Netherlands on: 27 June 2012

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EXECUTIVE SUMMARY

The Aquarius™-UV ballast water treatment system (BWTS) of the Hamworthy/Wärtsilä Corporation's Ship Power division was tested for IMO type approval at the facility of the NIOZ Royal Netherlands Institute for Sea Research from April to June 2011 and from March to May 2012. The Aquarius™-UV BWTS is a modular ballast water system with a treatment rated capacity of 250m3/h that is installed in bypass to the main ballast line. Treatment of ballast water is achieved through a two-step process. At intake the first step is filtration using a 40 µm super duplex screen. In the second step filtered water is directed into the disinfection chamber with a cross flow arrangement of twelve medium pressure ultraviolet lamps. After the ballast water has been stored, it is treated a second time at discharge with UV radiation only. In 2011 the Aquarius™-UV system was tested at 100% UV-power: four times at intermediate salinities and five times at high salinities. In 2012 the same system was tested at 60% (six times) and 100% UV-power (two times). In all tests the holding time before discharge was five days.

In general the G8 requirements for testing, that is the abiotic water quality and the abundance and biodiversity of organisms were met, although the extremely low freshwater discharge into the Wadden Sea in spring and early summer of 2011 led to lower than normal abundances of planktonic organisms. On the other hand, the overall biodiversity in the NIOZ test water was extremely high with a total of 17 different phyla in the 10-50 µm and >50 µm size classes and ca. 100 different species and species groups.

Treatment with the Aquarius™-UV system did not negatively change the abiotic quality of the discharge water. TSS and POC concentrations were reduced. Oxygen saturation levels were lowered but remained high enough to prevent local hypoxic conditions.

At the intermediate salinity regime at 100% UV-power the more than sufficient reduction of organisms in both size classes led to compliance with the D-2-standard. The total concentration of intact microzooplankton cells (10≤µm<50) in two tests performed at 100% UV-capacity in 2011 was slightly above 10 per mL. However, in 2012 it was shown in incubation experiments that this intact microzooplankton is not viable. Therefore, the total number of viable organisms in the 10≤µm<50 and >50 µm size classes in all six tests always met and exceeded the levels stipulated in the D-2-standard.

At the intermediate salinity regime at 60% UV-power the more than sufficient reduction of organisms in the 10≤µm<50 and >50 µm size classes led to compliance with the D-2-standard in five tests. The total number of viable organisms in the 10≤µm<50 and >50 µm size ranges in these tests always met and exceeded the levels stipulated in the D-2-standard.

At the high salinity regime at 100% UV-power the significant reduction of viable biological organisms led to compliance with the D-2-standard for >50 µm and 10≤µm<50 organisms in all five tests.

At all salinity regimes E. coli and Enterococci concentrations were below detection limits. Vibrio cholerae did not have to be tested because this organism is absent in NIOZ test water.

The biological efficacies at all UV-powers tested surpassed the combined D2-G8 requirement of 2.0 (10≤µm<50 organisms) and 4.0 (>50 µm organisms) with values of 2.6 to 3.3 and 4.5 to 4.6. These efficacies indicate a 2,000x (10≤µm<50 organisms) to 30,000x (>50 µm organisms) reduction where 100x and 10,000x are required.

In conclusion: during the land-based G8-tests at NIOZ the Aquarius™-UV ballast water treatment system fulfilled all requirements of the D-2 standard. The system performed very well at all salinities tested. Therefore, the configuration of the Aquarius™-UV system as tested at NIOZ in 2011 and 2012 is an environmentally safe ballast water treatment system with a high efficacy that meets and exceeds the reductions of viable organisms in the required size classes as stipulated in the D-2 Ballast Water Performance Standard.

SUMMARY TABLE. AQUARIUS™-UV BALLAST WATER TREATMENT SYSTEM

Organisms relevant for the D-2-regulation: *E. coli*, enterococci, and viable organisms in the size ranges 10≤µm<50 and >50 µm. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Total heterotrophic bacteria need to be measured at intake and discharge according to guideline G8, but are not regulated in D-2.

set I	control (T0)		control (T5)		treated (T5)		
	average	min-max	average	min-max	average	min-max	unit
Organisms > 50µm	101,000	50,000-148,000	50,000	34,000-71,000	2	1-6	per m3
Organisms 10≤µm<50	2300	1700-3000	110	100-130	0	0-0	per mL
Heterotrophic bacteria	470	124-762	187	27-327	138	56-277	x1000 per mL
E. coli	<10	<10	<10	<10	<10	<10	cfu/100 mL
Enterococci	<1	<1	<1	<1	<1	<1	cfu/100 mL

set II	control (T0)		control (T5)		treated (T5)		
	average	min-max	average	min-max	average	min-max	unit
Organisms > 50µm	107,000	50,000-130,000	45,000	34,000-54,000	3	0-5	per m3
Organisms 10≤µm<50	1800	1000-2700	90	10-130	4	0-6	per mL
Heterotrophic bacteria	373	124-563	34	20-48	145	66-255	x1000 per mL
E. coli	<10	<10	<10	<10	<10	<10	cfu/100 mL
Enterococci	<1	<1	<1	<1	<1	<1	cfu/100 mL

set III	control (T0)		control (T5)		treated (T5)		
	average	min-max	average	min-max	average	min-max	unit
Organisms > 50µm	58,000	32,000-88,000	27,000	15,000-42,000	0	0-1	per m3
Organisms 10≤µm<50	570	500-870	100	90-100	0	0-1	per mL
Heterotrophic bacteria	350	339-361	120	112-131	77	44-104	x1000 per mL
E. coli	<10	<10	<10	<10	<10	<10	cfu/100 mL
Enterococci	<1	<1	<1	<1	<1	<1	cfu/100 mL

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We also thank the Ph.D. students Isabel van der Star, Frank Fuhr and Cees van Slooten who contributed to the G8 tests.

Phytoplankton data of 2011 were obtained from dr. K. Philippart (In PLace). Phytoplankton data of 2012 were analysed by Koeman & Bijkerk, Groningen. Indicator bacteria in both years were analysed by C-Mark, Voorst. Parts of this report are based on previous G8-test reports by the NIOZ.

In addition we thank Dr. Ivan Lines and the team of Hamworthy engineers. Their cooperation was essential for the successful completion of the land-based tests.

We also thank Mr. M. Vink (Inspectie Leefomgeving en Transport/ILT, Ministerie van Infrastructuur en Milieu), acting on behalf of the Dutch Administration, for his collaboration during this whole certification process and Mr. T. Lupgens and Mrs. M.H. Hoikka of Lloyd's Register who inspected the tests in 2011 and 2012, respectively.

1 INTRODUCTION

Ships transport five to ten billion tons of ballast water annually over the globe (Endresen et al. 2004). This ballast water is loaded with particulate sediment and an enormous variety of living organisms ranging from juvenile stages, larvae and eggs of fish and larger zooplankton (Williams et al. 1988, Carlton & Geller 1993) to macroalgae, phytoplankton (Hallegraeff et al. 1997, Hamer et al. 2000), bacteria and viruses (Gollasch et al. 1998) . In general these organisms belong to the natural ecosystem in and around the port of origin but they might not be occurring naturally in the coastal waters and port of destination at the end of a ship's voyage. In hundreds of cases around the world, this has resulted in severe damage to the receiving ecosystem and to human health, because several of these non-indigenous organisms developed into a plague. This can have a high impact on the natural ecosystem and can cause significant ecological and economical damage (Hoagland et al. 2002) , when it results in a decrease of stocks of commercially valuable fish and shellfish species. Occasionally outbreaks of diseases such as cholera can also occur (Ruiz et al. 2000, Drake et al. 2001). If no action is taken, the problem of invasive species may increase dramatically for several reasons. Ships are getting larger, faster and the amount of traffic across the oceans is expected to increase rapidly during the coming decades. This results in an increased volume and transfer rate of ballast water and, therefore, also an increased chance of non-indigenous organisms to have large enough numbers for settling and expanding. Efforts to reduce pollution of ports and coastal waters have also improved the quality of the aquatic environment in these areas but this increases the susceptibility to invasive organisms. The problem of invasive species is considered as one of the four major threats of the world's oceans next to land-based marine pollution, overexploitation of living marine resources, and physical alteration/destruction of habitats.

To minimize these risks for the future, the International Maritime Organization (IMO) of the United Nations has adopted the Ballast Water Management Convention (BWMC) in 2004 (IMO 2005). The Convention states that all ships (>50,000 in number) should install proper ballast water treatment (BWT) equipment on board between 2009 and 2016. Although at present the number of countries ratifying the Convention has reached the required minimum, the required tonnage has not. Yet, the expectation is that the Convention will be implemented in the near future.

An overview of the current status and upcoming challenges for the implementation is given in the proceedings of the IMO-WMU Research and Development Forum of the meeting held in Malmö (Sweden, January 2010).

As a temporary solution ships may reduce the risk of invasive species by performing ballast water exchange during their voyage when passing deep water (>200 m depth and 200 NM from the coast). Ballast water exchange faces many problems as to feasibility, safety and efficacy. For a large part of ships' voyages the required depth and/or distance to shore requirements are not met. Ballast water exchange can affect a ship's construction stability and in rough seas exchange is not possible because of the risk to ship and crew. Treatment of ballast water is therefore considered to be the best solution of reducing the risk of invasive species.

During recent years numerous solutions for treatment of ballast water have been mentioned and tested with the ultimate goal to reduce the amount of organisms in ballast water (Tsolaki & Diamadopoulos 2010, Goncalves & Gagnon 2012). However, next to a high efficacy there is more needed for a BWT system to be a good system. Next to being biologically effective the system should be practicable, environmentally acceptable and also cost effective. Besides reducing the load of organisms the sediment load should be reduced as well.

Role of NIOZ in ballast water research and certification of BWTS

NIOZ is the national oceanographic research institution of the Netherlands. In the course of general oceanographic research, several methods have been developed to count and characterize different classes of planktonic organisms, including viability, and a large number of abiotic variables. This set of analytical methods also forms the core of the measurements required for the land-based tests which are part of the requirements for the certification of BWTS according to IMO-guidelines. The NIOZ head-office at the island of Texel is located at the Marsdiep tidal inlet on the border of the western Wadden Sea and the coastal North Sea; which is a highly productive shallow sea area with a large variety of natural plankton. NIOZ has been certified by Lloyd's Register for this purpose. The main tests are carried out in the NIOZ harbour and the analyses partly directly at the harbour and partly at the well-equipped laboratories of the nearby institute. The ballast water research group is embedded in the scientific department of Biological Oceanography. More details follow in the next chapters. Besides the certification testing, the group is also involved in further method development for land-based and shipboard testing, and compliance monitoring and enforcement by the responsible authorities.

2 GENERAL DESCRIPTION

Figure 2.1. Aerial view of the NIOZ harbour (lower right), NIOZ and the TESO ferry connecting the island of Texel with the main land (top). The Mokbaai is the source for additional suspended solids.
©Photo: Simon Smit Photography, Den Burg, Texel.

2.1 NIOZ profile

NIOZ Royal Netherlands Institute for Sea Research is the National Oceanographic Institute of the Netherlands. NIOZ is an institute of the Netherlands Organization for Scientific Research (NWO). The institute employs about 340 people at locations on the island of Texel on the border of the North Sea and the Wadden Sea (main location) and in Yerseke in the southwest of the country. The annual budget is approximately €30 million.

The mission of NIOZ is to gain and communicate scientific knowledge on coastal seas and oceans for a better understanding of the system and sustainability of our planet, to manage the national facilities for sea research and to support research and education in the Netherlands and in Europe.

In order to fulfil its mission, the institute performs tasks in four specific fields.

Research: The emphasis is on innovative and independent fundamental research in continental seas and open oceans. Increasingly, the institute also carries out research based on societal issues. The senior scientists at NIOZ all participate in international research projects. Several of them also hold a professorship at Dutch or foreign Universities.

Education: The institute educates PhD students and master students of universities and schools for professional education. Together with several universities, NIOZ also organises courses for PhD students and master students in the marine sciences. A number of our senior scientists is also appointed as professor at Dutch and foreign universities.

Marine Technology: NIOZ has its own workshops for mechanical, instrumental en electronical engineering. Here, marine research equipment is being designed and built according to the wishes of our individual scientists.

Facilities: NIOZ invites marine scientists from Dutch and foreign institutes and universities to write scientific proposals involving the institute's research vessels, laboratories and large research equipment. Our ocean-going research vessel 'Pelagia' is shared on a European level in the 'Ocean Facilities Exchange Group' (www.ofeg.org).

The basic scientific disciplines at NIOZ are physics, chemistry, biology and geology. Multidisciplinary sea research is regarded as one of the main strengths of the institute. Therefore, the research is organised in 5 multi-disciplinary themes: 'Open ocean processes, Sea floor dynamics, Wadden and shelf sea systems, Climate variability and the sea and Biodiversity and ecosystem functioning'.

Together with a number of oceanographic partners, NIOZ also maintains the popular marine website www.seaonscreen.org.

For more information, please contact our Communication & PR department at cpr@nioz.nl, or visit our website at www.nioz.nl.

NIOZ has extensive experiences in the field of ballast water and ballast water treatment technologies at its harbour on the island of Texel. During the past seven years several pilot tests for ballast water treatment were conducted in the NIOZ harbour and so far, between 2007 and 2010 seven full scale land-based tests were carried out for Final and Type Approval.

2.2 North Sea Ballast Water Opportunity project

From 2009 onwards the activities of NIOZ in ballast water research has been organized in a broader framework, the North Sea Ballast Water Opportunity project (www.NorthSeaBallast.eu). This project was an initiative of the BSH (Federal Maritime and Hydrography Agency, Germany) and NIOZ and involves all relevant stakeholders within the maritime sector in the North Sea region: governmental institutions, inter-governmental and non-governmental organisations, industry and scientific and technological institutes. This structure and participation offers a broad and sound base for the project in support of a successful implementation of the IMO Convention in the region. Moreover, the project being one of the largest and most integrative in its kind, the objectives (investments) will become available as a model for other European maritime regions as well as other regions across the globe. To facilitate this initiative, funding was received from the North Sea Interreg IVB (an ERDF program). For the embedding in a more global strategy the project is liaising with the Globallast II initiative of the IMO and currently involves also comparable research initiatives in the US (GSI, MERC and Golden Bear).

2.3 NIOZ test facility

The land-based tests were carried out in the NIOZ harbour on the island of Texel from April to June 2011 and March to May 2012 on the Pelagia quay where three coated tanks of 300 m³ simulate ship's ballast water tanks. The tanks were cleaned with high pressure steam after each test. Water samples can be taken from bypasses of the standard piping (DIN 200) used to fill and to empty the tanks (Figure 2.2). According to the requirements of the Guidelines G8, sampling points are fitted directly after the ballast water pump, and directly after the BWTS.

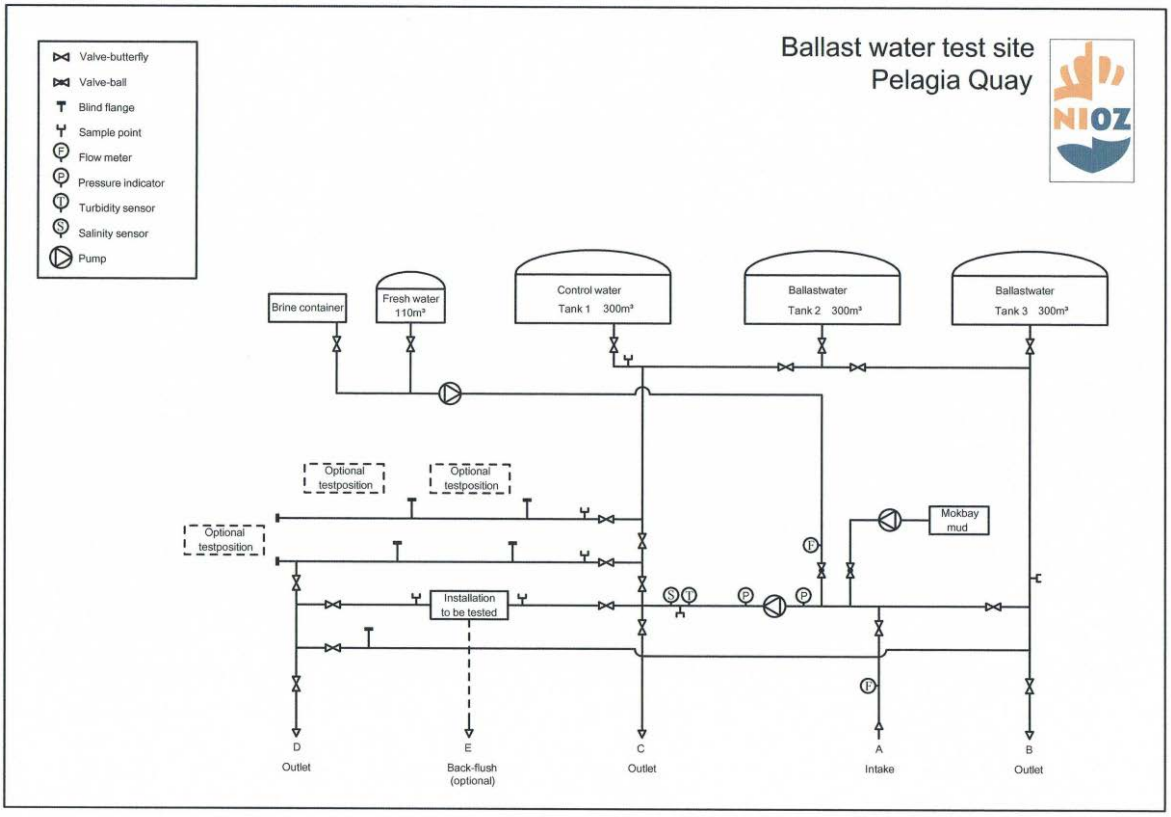

Figure 2.2. Piping and Instrumentation diagram of the Pelagia quay test site at the NIOZ harbour. The installation tested was a UV-treatment system. The installation consists of three ballast water tanks, one for control (untreated) water and two for treated water.

The Hamworthy Aquarius™-UV system was connected to a water pump (capacity of up to 500 m^3/h) which was located in the NIOZ harbour. This is a pristine harbour with a direct access to the Wadden Sea and the origin of the test water changes with the tide. Furthermore, provision were made to allow the addition of brine water and/or freshwater in order to adjust the salinity of the natural water of the NIOZ harbour with ±2 PSU to the required test conditions of brackish water and marine water with a minimum of 10 PSU difference. A detailed description of the test installation is presented in Figure 2.2.

2.4 Profile of Hamworthy Water Systems Ltd.

Hamworthy is an innovative, market leading, global company providing high technology products, systems and services to the marine and oil & gas industries. It produces technically advanced solutions, often in response to environmental needs and legislation. Hamworthy Water Systems, a division of Hamworthy plc, provides a range of water treatment systems including ballast water systems, sewage treatment plants, grey and black water treatment, freshwater generators and condensation plants. Since February 2012 Hamworthy forms part of the Wärtsilä Corporation's Ship Power division.

2.5 Technical Overview of the Aquarius™-UV system

Aquarius™-UV is a modular ballast water management system with a treatment rated capacity of 250m^3/h (test unit, Figure 2.3). The system is installed in bypass to the main ballast line provides a safe, flexible and economical process for the treatment of ballast water and eradication of aquatic invasive species. Treatment of ballast water is achieved through a simple and efficient two-step process:

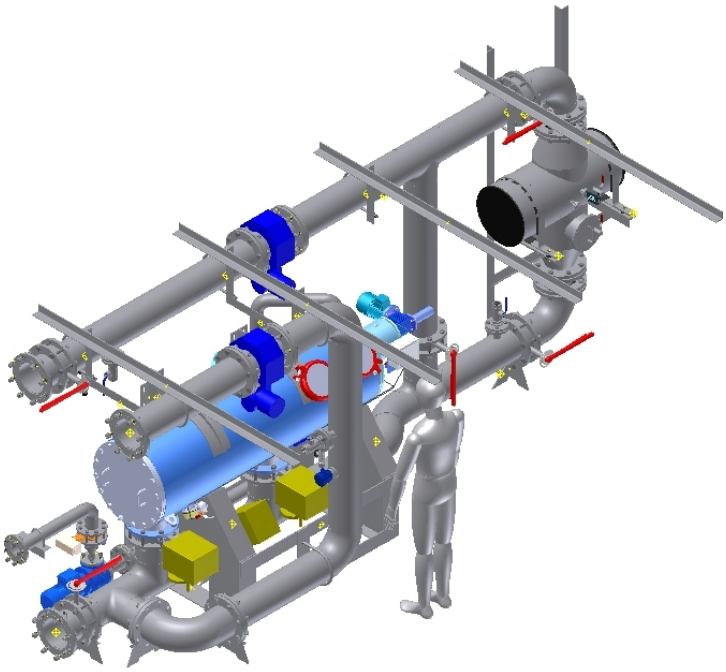

Figure 2.3. Aquarius™-UV BWTS test unit.

The first step of the process is filtration (Figure 2.4). Ballast water passes through an automatic back flushing BallastSafe™ filter capable of removing particulates, sediments, zooplankton and phytoplankton using a 40 µm super duplex screen. The automatic filter cleaning cycle is activated when the pressure drop across the filter reaches 0.5 barg (gauge pressure in bar). This ensures that the screen is kept clean and the filtration process maintained at maximum efficiency at all times. An alarm is triggered if the differential pressure reaches 0.8 barg. The filter backwash flow is in the range 1-5% of the total flow and is discharged overboard at the ballasting location. The backwash flow range depends on the quality and composition of the water drawn into the system.

Filtered ballast water is directed into the disinfection chamber (Figure 2.4) where a cross flow arrangement of twelve medium pressure ultraviolet lamps treats the incoming ballast water before it enters the ballast tanks. The UV light intensity is continuously monitored during system operation to make sure that the intensity is maintained above pre-set values and to ensure the delivery of the required dose. The UV-lamps are housed within quartz sleeves. An integral and automatically activated sleeve wiper cleaning mechanism minimises bio-fouling and controls the accumulation of deposits on the UV lamp sleeves.

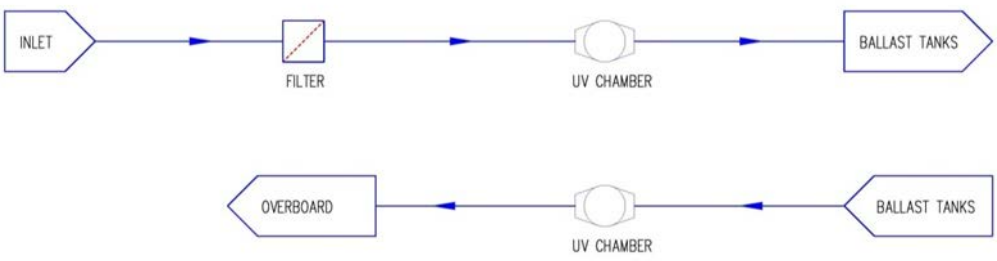

Figure 2.4. Aquarius™-UV BWTS block process diagram.

At discharge, the filter is by-passed and the ballast water is pumped from the tanks through the UV disinfection chamber (Figure 2.4). Thus, the ballast water is subjected to a second UV disinfection treatment prior to discharge.

According to the manufacturer, specific features/advantages of Aquarius™-UV above other BWT-systems are:

- Wide environmental operating envelope
- Modular construction, suitable for new builds and retrofit
- Flexible up-scaling
- Intelligent PLC control ensuring safe and economical operation
- Automatic dose regulation
- No use of active substances
- Integrated antifouling control system (no cleaning in place, CIP)

2.6 General test set-up: treatment and control tanks

A typical land-based test of a treatment system is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day at approximately the same phase in the tidal cycle. The control tank with untreated water serves as reference to examine the effect of the treatment, including holding for at least 5 days (§2.3.35 G8-guidelines). The control tank can also indicate an unexpected source of mortality due to the testing arrangement (§2.3.37 G8-guidelines). Therefore, the average discharge results in the control water should not be less than or equal to 10 times the values mentioned in regulation D-2.1 (§2.3.36 G8-guidelines) for treated ballast water.

The number of tests to be performed is five at intermediate salinity and five at high salinity. NIOZ will report the total number of tests that were needed to meet the D-2-standard five times for each salinity range.

Samples varying in volume from 1 L up to 1 m^3 (IBC's) were taken using clean sampling containers. Sampling containers and all further handling of the samples were separated in a control and a treated set to avoid cross contamination. The basic handling, such as the concentration of organisms ≥50 µm and filtration was done directly at the NIOZ harbour. Different samples (1 to 10 L) were transported to the institute's laboratories for further special analysis. For re-growth experiments 10 L of sample was transported in a polycarbonate Nalgene bottle to a climate room for incubation experiments (ca. 10 – 15 °C; a light:dark regime of 16:8 h and 100 µmol quanta m^{-2} s^{-1}).

3 D-2 and G8 requirements

3.1 D-2 requirements

According to the D-2 Standard of the IMO/MEPC Convention of 2004 (IMO 2005, 2008) ships that meet the requirements of the Convention by meeting the ballast water performance standard must discharge a maximum of organisms mentioned in Table 3.1.

Table 3.1. Ballast Water Performance Standard Regulation D-2 of the International Maritime Organisation. Organisms ≥ 50 µm are mostly zooplankton. Organisms 10 ≤µm<50 contain phyto- and microzooplankton. *V. cholerae*, *E. coli* and intestinal enterococci are indicator bacteria used as a human health standard.

Organism	Concentration	Remark
≥ 50 µm	<10 per m^3	size as minimum dimension and viable
10 ≤µm<50	<10 per mL	size as minimum dimension and viable
Vibrio cholera (O1 and O139)	<1 cfu/100 mL or <1 cfu/g wet zooplankton	cfu = colony forming unit
E. coli	<250 cfu/100 mL	cfu = colony forming unit
Intestinal enterococci	<100 cfu/100 mL	cfu = colony forming unit

The D-2 Standard is defined as a standard for the water characteristics at discharge. It contains biological variables only. However, with the exception of some indicator microbes (point 3) organisms < 10 µm are excluded from any further consideration.

The D-2 Standard is clear with respect to the maximum number of remaining viable organisms. On the other hand the definition of the dimensions of organisms is ambiguous. The 'minimum dimension', as in D2.1 and G8 § 2.3.20, is usually interpreted as the smallest of two dimensions when the organism is seen microscopically, i.e. by observing length and width (G2 § 3.1.1). However, the thickness of the organism, its third and actual minimum dimension, which is smaller than its length and width, can sometimes not be accurately microscopically observed. Theoretically and assuming laminar flow, the second dimension (width) will determine if an organism will pass a 10 or 50 µm two-dimensional sieve (§ 2.3.31 and 2.3.32). So, the IMO definition of 'minimum dimension' could practically be considered as an operational definition. An extended definition of the minimum dimension is given in 3.2.3.

Moreover, the IMO states (§ 4.6) that an organism's viability 'can be determined through live/dead judgement by appropriate methods including, but not limited to: morphological change, mobility, staining using vital dyes or molecular techniques'. The problem here is that viability is in fact the ability to reproduce while methods such as assessing mobility or vital staining indicate if an organism is vital, i.e. live or dead (Peperzak & Brussaard 2011). Vitality measurements are fast methods that typically take less than 1-2 hours. Viability measurements take much longer, typically > 1 day, because the reproduction of organisms is a relatively slow process. In fact, samples would need to be incubated under laboratory conditions that are representative for the test water's abiotic characteristics (light, temperature) and the reproduction of the organisms needs to be assessed on a daily basis. A complicating factor is that these viability measurements can be performed relatively easily for unicellular organisms that may divide once per day, but for multicellular organisms such as mesozooplankton that have complicated life cycles (e.g. including eggs), these are highly impractical. Therefore, the IMO definition of 'viable' is usually interpreted as 'vital'.

When UV radiation is applied in a treatment their DNA is damaged. As a result they can no-longer reproduce, but they may not have suffered morphological damage and may still appear vital. Therefore, NIOZ has adopted a variety of methods to determine the vitality and viability of different types of organisms. The vitality measurements include morphological

change, mobility and vital dyes for both uni- and multicellular organisms. The viability assessments of the unicellular organisms consist of incubations of discharge samples.

3.2 Guidelines (G2, G8, G9)

Next to the D-2 Standard, several guidelines were developed by the IMO as a framework for approval of ballast water treatment systems (G8) and approval of the use of active substances in ballast water treatment systems (G9). Guideline G2 gives specific definitions of the minimum dimension of organisms, including colony forming species. For land-based testing MEPC 53/Annex 3 (IMO 2005) and modifications as adapted at MEPC 174.58 (IMO 2008) was compiled of which the most relevant parts will be presented below. These guidelines were generically designed to meet the conditions of a broad range of potentially effective treatment techniques to be tested in typical port and environmental conditions found across the globe. The land-based tests serve to determine the biological efficacy of the BWT systems under consideration for Type Approval under more or less controlled and replicable conditions. The approval testing aims to ensure replicability and comparability to other treatment equipment (§ 2.3.7). The implications of using natural test water of varying abiotic and biological quality on replicability and comparability will be discussed later in this chapter.

3.2.1 Abiotic quality requirements

Table 3.2. Abiotic requirements in test water according to the G8-guidelines.

	Salinity range			units
Salinity	> 32	3 – 32	< 3	PSU
Total Suspended Solids (TSS)	> 1	> 50	> 50	mg/L
Particulate Organic Carbon (POC)	> 1	> 5	> 5	mg/L
Dissolved Organic Carbon (DOC)	> 1	> 5	> 5	mg/L

One of the main criteria in the G8 test requirements is the salinity range and related to this the differences in Total Suspended Solids (TSS), Particulate Organic Carbon (POC) and Dissolved Organic Carbon (DOC). This resulted in three main categories of test conditions (Table 3.2).

In general UV-reactors are not sensitive to changes in the salinity of the water except that waters with a high alkalinity, iron and/or manganese concentrations influence the transmittance of the water and can, depending on the UV sleeves cleaning technology, increase fouling of the UV-light source (Gundry 2007). Historically, UV-reactors are widely used in treating drinking and sewage waters and their use as a tool for disinfection is well studied. It was for this reason that the Type Approval tests were conducted at the intermediate (3 – 32 PSU) and high salinity (>32 PSU) regions. The difference in composition of the test water between the freshwater and intermediate salinity water is the presence or absence of (sea)-salt. All other minimum requirements for TSS, POC and DOC for these two water types are identical (Table 3.2).

A further requirement is that the difference between the two salinity regimes should be at least 10 PSU. The test water, originating from the Wadden Sea, and the actual sampling varies with the tide at the NIOZ test facility and as a result salinity was subject to variations. To enhance a salinity differences between the test regimes, freshwater was added to low salinity Wadden Sea test water, and the salinity of coastal North Sea water was increased by adding a brine solution of commercially available salt. These additions were made close to the pump site, to ensure proper mixing, with a constant flow rate.

3.2.2 Biological quality requirements

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by G8 to guarantee the effectiveness of the BWTS in different ecosystems. The diversity of organisms in the test water is essential in order to demonstrate that the BWTS can effectively deal with the biodiversity that could be encountered across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 3.3.

Natural water, originating from the coastal zone of the North Sea (high tide) and the inner Western Wadden Sea (low tide) was used. The test period covered the whole spring and early summer of the plankton growth season and therefore includes the natural occurring biodiversity and species succession. The ambient plankton content in terms of species diversity in the relevant size classes is very high. For instance, in 2011 16 phyla and more than 70 species were detected during the 2011 test season (Table 5.4), where only five species and three phyla are required (§ 2.3.20 of G8).

Table 3.3. Biological requirements in test water according to the G8-guidelines. 1 µm = 1 micron = 0.001 mm.

Intake test water		
Organism	unit	Variety
≥50 µm	> 10^5/ m^3	at least 5 species from at least 3 different phyla/divisions
≥10 and <50 µm	> 10^3/ mL	at least 5 species from at least 3 different phyla/divisions
heterotrophic bacteria	> 10^4/ mL	not further defined

The natural waters of the test area include a large range of organisms varying in sensitivity to mechanical stress, UV radiation or various active substances used in ballast water treatment. Besides fragile organisms also plankton that is highly adapted to harsh environmental conditions, mostly hard shelled organisms, are present in the test water. Therefore, the test water at the NIOZ facility provides a significant challenge to the BWTS tested due to the rich organism diversity in the natural waters at this locality.

For completeness, the plankton fraction <10 µm is also included in the NIOZ analyses although this is not required by the G8-guideline and the D-2 standard.

3.2.3 Minimum dimension of organisms

The ‘draft guidelines for ballast water sampling (G2)’ provides a definition for ‘minimum dimension’ in § 3.1 (G2). Here, the minimum dimension is defined as the smallest dimension between main body surfaces of an individual when looked from all perspectives. As argued in the paragraph on D-2 requirements above, this is usually not possible and very unpractical.

G2 § 3.1 also states that ‘for colony forming species, the individual should be measured as it is the smallest unit able to reproduce that needs to be tested in viability tests.’ The statement ‘viability tests’ is unclear because, according to D-2, viable organisms have to be counted. The present NIOZ interpretation from 2011 onwards is that according to G2 individual viable cells of a large colony should not be counted when they are smaller than 10 µm, as they are not part of D-2.

In practice, this means that the colonies of the phytoplankton genus *Phaeocystis*, with individual cells that are <10 µm diameter, are not counted. Because the *Phaeocystis*

colonies are usually much larger than 50 µm in diameter, in the past their numbers were included in the test water counts in the >50 µm category. A discrepancy in large organism concentrations may therefore be apparent between reports prior to 2011 and the present one.

3.2.4 Human pathogens

Within the group of prokaryotic microbes only heterotrophic bacteria (Table 3.3) have been taken into account by the D-2-standard, but for completeness D-2 should include all bacteria and also Archeae. While these microbes are part of the natural community in the aquatic environment the indicator microbes (Table 3.1), i.e. the human pathogens, are introduced as part of human activity and often associated with discharge of sewage. In the present research all microbes have been included as a bulk parameter, the number of heterotrophs as a viable component as well as the viability of the whole microbial community has been determined.

Within the whole microbial community the number of heterotrophic bacteria was determined as well as *E. coli* and total enterococci. The test area of the institute is part of a tidal estuary of the Wadden Sea, which is essentially a pristine environment. Moreover, waste water treatment is highly developed in the Netherlands. Therefore, numbers of these human pathogens during the tests were expected to be low. *V. cholerae* is not present in the region; therefore no samples were taken to determine the presence of this pathogen.

3.3 NIOZ approach to testing with a naturally variable water quality

In addition to ambiguities or omissions in the IMO convention (organism size, viability/vitality, <10 µm organisms) the use of natural water poses a number of challenges that need further evaluation. Natural waters, especially from coastal regions as the North and Wadden Sea provide an excellent opportunity to tests BWT systems under relevant conditions of abiotic and biological variables. However, this relevancy also implies that the test conditions vary and that replicability and comparability with other test facilities and other treatment equipment will decrease. In other words, replicability and comparability would benefit from tests performed under nearly identical abiotic and biotic circumstances, including a standardised biodiversity. On the other hand, testing under nearly identical and artificial circumstances would seriously reduce the relevancy of the tests.

Testing at NIOZ under relevant naturally fluctuating environmental conditions also implies that tests may not always comply with the IMO G8-guidelines. Meteorological forcing such as high rainfall or strong gales may influence abiotic variables such as salinity and biological variables such as zooplankton abundance. Furthermore, high test water concentrations of mesozooplankton that graze upon algae may lead to low phytoplankton concentrations. This natural variability is hard to predict and can only be responded to by the test facility to a certain degree as not to jeopardize the quality of the test water. For instance, NIOZ can adjust salinity, TSS and POC by adding freshwater, brine or mud, but the amount of for instance freshwater that can be added before killing marine organisms is off course limited. Furthermore, NIOZ does not add cultured organisms such as *Artemia* because these large animals are easy to remove by filtration, so they do not add to the quality of the tests. Moreover, non-indigenous species cannot be released into the Wadden Sea at discharge, especially in the case of untreated control water. Adding concentrated naturally occurring organisms appears to be an option to increase the concentrations of phytoplankton and zooplankton. However, it must be realised that concentrating zooplankton, as can be done with a plankton net, will take a long time, the animals will be damaged in the net and their enhanced abundance may lead to increased self-predation. In addition, by the time that a considerable amount of organisms has been collected, their physiological status will be impaired and the quality of their addition to the test water may be seriously doubted.

In this paragraph the NIOZ interpretation of BWTS testing will be given in scientific terms and in relation to D2 and G8. In particular it is argued that:

1. BWT systems tests can be performed as scientific experiments (G8 §2.3.35) using appropriate statistical analysis
2. The Ballast Water Performance Standard (D2) determines if a test passes or fails
3. G8 contains guidelines for testing, not absolute rules
4. For each set of test cycles (salinity range) the minimum biological efficacies of a BWT system should comply with the equivalent to G8 divided by D2 (G8 §2.3.20.1/D2.1)
5. The quality of testing is improved if the total number of phyla and species to which a BWTS has been subjected is more than advised in G8.

3.3.1 BWTS tests as scientific experiments

An experiment in which a certain treatment is examined should be compared to a control experiment in which this treatment is not applied. Although counterintuitive, the scientific hypothesis tested is: there is no difference between treatment and control. This 'no difference', null hypothesis (H_0) is fundamental. One might expect an effect of a certain treatment but the scientific goal is not to prove this expectation. By measuring a set of variables in both the treatment and the control during an experiment and by applying an appropriate statistical test to the experimental data two outcomes are possible: 1) the null-hypothesis is not rejected, i.e. there is not enough difference between control and treatment, or 2) the null-hypothesis is rejected because there is actual evidence that the treatment data are not by chance different from the control. The chance that the zero-hypothesis is rejected incorrectly is usually set at 5% (e.g. $P<0.05$). If the zero-hypothesis is rejected, the alternative hypothesis becomes true: there is a significant difference between treatment and control.

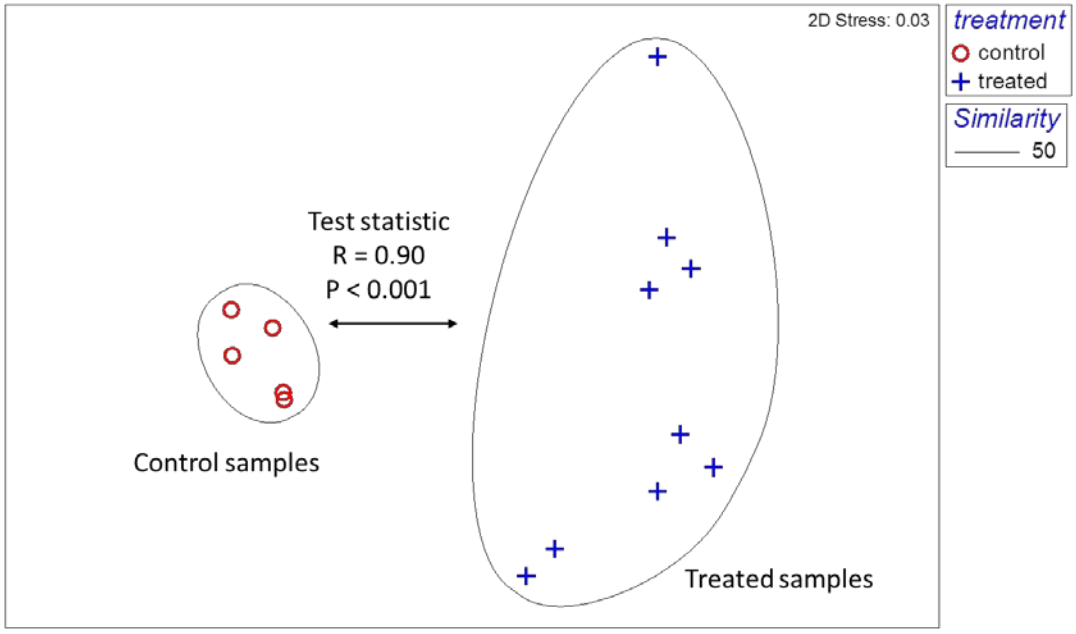

Figure 3.1. Example of a multivariate one-way ANOSIM test result in a non-parametric multi-dimensional scaling (NMS) diagram. The control samples (left, after five days holding time) are significantly different from the filter+UV-treated samples (right, after five days holding and the second UV-treatment at discharge). This statistical analysis was based on the concentrations of total phytoplankton (10≤µm<50), microzooplankton (10≤µm<50) and mesozooplankton (>50 µm). The test statistic R is highly significant ($P<0.001$) and therefore the null-hypothesis (control = treatment) is rejected. In other words, the treatment delivers significantly different results compared to the control.

NIOZ uses a multivariate statistical test to investigate the null-hypothesis that the organism abundances in treated water and in the control water are equal. This means that three variables, phytoplankton, microzooplankton and mesozooplankton concentrations are tested

simultaneously for multiple of tests. Because the concentrations of the pathogenic bacteria in NIOZ test water are always below the detection limits, they are not included in the statistical test, which is a one-way ANOSIM (analysis of similarities) permutation test. The test itself is described in more detail in § 4.7. Figure 3.1 gives an example of an ANOSIM test result performed on data from 2011 of the Aquarius™-UV system.

3.3.2 D2 determines pass or fail

Although a multivariate statistical test may indicate a significant difference between multiple ballast water treatments and their controls (Figure 3.1), the fail or pass of any given test is based on the fulfilment of the Ballast Water Performance Standard (D2). A concentration of ≥ 10 viable organisms (10≤µm<50 per mL or >50 µm per m3) will still fail an individual test.

3.3.3 G8 contains guidelines, not absolute rules

The difference between the Ballast Water Performance Standard (D2) and G8 is that the latter in NIOZ' opinion is what it says: a guideline. In other words, when using natural water G8 provides leeway to test at concentrations that are not always according to the specified numbers. For instance, the organism >50 µm concentration of 105 m-3 is set so high that these are difficult to reach in all circumstances. Some test facilities have therefore decided to add cultured organisms such as Artemia or oyster larvae in order to reach such a high abundance. As explained in § 3.2.5 NIOZ has good arguments not to add non-indigenous species or concentrated indigenous ones to its test water.

Abiotic factors such as salinity or DOC may also not always be according to G8. Salinity in coastal waters is very dependent on river discharge and in dry spring seasons with little rainfall the test water salinity might be so high that it cannot be reduced with freshwater enough to achieve a 10 PSU difference with high salinity test water. In addition, DOC concentrations are relatively independent of salinity which means that there is little difference in DOC between intermediate and high salinity tests.

Depending on the principle operating technique of the tested BWTS it can be argued that deviations from the G8 guidelines are permissible. A system that depends on naturally available salinity to produce an active chlorine-based substance should be tested at a wide variety of salinities. On the other hand, for a UV-system for instance, salinity has no fundamental influence. It could be argued that it would be better if a UV-BWTS was tested at a range of UV-T values instead of different salinities. For an active chlorine-based substance the total amount of organic carbon in the test water is of importance, i.e. the sum DOC and POC (TOC), not DOC alone. In other words, valid and meaningful tests are possible in test water deviating from the G8 guideline. In future, additional specific test conditions could be devised for particular BWT systems.

3.3.4 Efficacies of a BWTS should be ≥ 2.0 and ≥ 4.0

"The land-based testing serves to determine the biological efficacy and environmental acceptability of the BWMS under consideration" (§ 2.3.7 G8). The efficacy of a BWTS can be defined as the ratio between the G8-intake concentration of an organism and its intended reduction to comply with D2. In the case of >50 µm organisms for instance, this means that a concentration of 100,000 per m^3 needs to be reduced to <10 per m^3, which is a reduction of 10,000x or in logarithmic terms an efficacy of 4: $\log_{10}$ (10,000) = 4.0. This is graphically demonstrated in Figure 3.2. In the case of 10-50 µm organisms the efficacy should be reduced from 1000 per mL to <10 per mL, which is an efficacy of 2.0. The formulas for calculating the efficacies of the two size groups of organisms are given in 4.7.2.

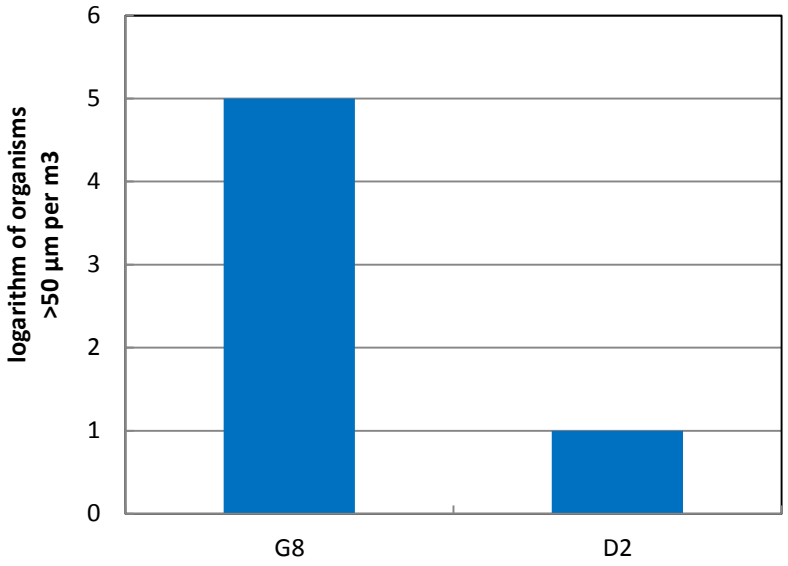

Figure 3.2. The minimum BWTS efficacy for organisms >50 µm that should be reached according to G8 and D2 is the difference between the logarithms of their concentrations which is: 5 - 1 = 4. It can be argued that an efficacy of ≥4 can also be achieved when the test concentration is lower than 10^5 per m^3, e.g. 8 x 10^4 (80%). In that case the concentration after treatment should be < 8 per m^3.

Adapting efficacy as a leading principle in BWTS testing does not mean that testing becomes easier for facilities that are dependent on natural testing water. The price for testing slightly lower concentrations than advised by G8 is a more stringent application of D2.

3.3.5 Biodiversity and the quality of testing

The biodiversity of the test water should be such that at least five species from three phyla should be present (§2.3.20 G8). NIOZ uses the on-line World Register of Marine Species (WoRMS, (Appeltans W et al. 2012)) for the classification of the species that were found during BWT tests. This register lists over 30 phyla of marine animals, indicating that only a minority of the major taxonomic groups needs to be tested. On the other hand, the natural biodiversity in NIOZ test water that is taken from the Wadden and North Sea is much higher. In 2011 for instance 10 phyla of organisms >50 µm were present in the test water. Although this number of phyla is still lower than the theoretical maximum, the testing of three times more phyla than required by G8 presents a far more realistic scenario for BWT systems as these are likely to be employed around the world. In other words, the quality of the test is considerably enhanced by a high biodiversity.

At present it is not needed to make a distinction between tests performed at a relatively low, three phyla and five species, and a relatively high biodiversity. Neither is it necessary to account for the use of easily-removable cultured organisms or the use of physiologically impaired natural organisms that were concentrated and added to the test water to top up the natural concentration of organisms to the appropriate but unrealistic G8 guideline. In evaluating the overall BWTS test results the biodiversity of the test water and, therefore, the quality of the tests should play an important role.

4 Experimental design

A variety of methods was applied to examine the biological efficacy of the Aquarius™-UV system for the different categories of organisms during the two test series. A detailed description can be read the in outline of the official test protocol for the Aquarius™-UV system. Sample handling and volumes were according to the description of the guideline for BWT testing (G8) or they have been described in detail when these guidelines were insufficient or when other considerations were taken into account, e.g. in the case of sampling and incubation of samples at discharge.

4.1 Test design at intermediate and high salinity

A typical test of a treatment system is performed with two treatment tanks and one control tank that are filled in rapid succession, i.e. on the same day approximately within four hours in the same period of the tidal cycle. After the first treatment test the whole BWT system is shut down according to the manufacturer's procedure. Subsequently, the control tank is filled immediately after the first test run by only pumping water in the tank. The second treatment test run, starting with a complete starting up procedure, is performed after the control tank has been filled. This means that the two tests share the same control but they are sampled independently.

Two sets of test cycles should be performed by the Aquarius™-UV system, five test cycles or replicates at intermediate salinity and five at high salinity. Early in 2011 testing was carried out to identify system settings that would deliver compliant results. These early tests were carried out with the system operating at 50 and 75% of maximum design lamp power. Fully compliant D-2 results were obtained from these test runs. The first two tests at intermediate salinity were performed at these lower power levels and were followed by a further four tests using 100% of the maximum design lamp power (Table 4.1). The high salinity tests were all performed at 100% power of the UV installation (Table 4.1).

Following completion of two test cycles at intermediate and high salinities in 2011, the system continued to be tested that year in order to verify the optimised dose level that would deliver D-2 compliance. This testing was carried out using the same BWTS as used in the previous tests, i.e. the system configuration was unchanged and there were no changes or replacements of any parts. As a result, the UV-control system was adjusted in 2012 to operate at a lower design lamp power of 60%, the optimum condition for Aquarius™ UV.

Discussions with the Flag Administration of the Netherlands (ILT), concluded that a minimum of four additional tests in 2012, with a lamp power of 60%, would be required in order to address the G8-requirements. These additional tests, whose number was eventually even increased, were performed at the most challenging set of salinity conditions possible: the intermediate salinity range with increased TSS. Furthermore, NIOZ advised to include two tests at 100% power back to back with two of the four 60% power tests in order to allow a direct comparison between the two operational settings. This test series was executed in March and May 2012 (Table 4.1).

In all, six tests were performed at 60 and 100% (intermediate salinity) and five tests at 100% (high salinity) (Table 4.1).

Table 4.1. Three sets of test cycles performed by the Aquarius™-UV system in 2011 and 2012. Roman numerals were used in the original NIOZ data files. Sequential numbering is used in this report.

	Set I	Set II	Set III
Salinity	intermediate	intermediate	high
UV-power	100%	60%	100%
2011	VII (1)		XVII (1)
2011	VIII (2)		XVIII (2)
2011	XI (3)		XXI (3)
2011	XII (4)		XXII (4)
2011			XXIII (5)
2012	VI (5)	III (1)	
2012	VIII (6)	IV (2)	
		V (3)	
		VII (4)	
		IX (5)	
		X (6)	

4.2 General sampling strategy

Samples are generally taken:

1. In the harbour to assess test water quality before the pump. Harbour water samples are analysed regularly from February onwards in order to monitor the spring plankton bloom. Due to a lack of harbour water data on >50 µm organisms in 2011 their concentrations were calculated from untreated control samples and a known reduction percentage of the ballast pump.
2. Immediately before the treatment equipment from the main pipeline but after the ballast pump that is used to pump up the test water from the harbour (control, T0).
3. Immediately after treatment from the main pipeline (treated, T0) and
4. During discharge from the main pipeline, after the pump, after 5 days (control and treated, T5) holding time (§ 2.3.2 and 2.3.26 G8-guidelines) and after completing a second passage through the BWTS when this step forms part of the treatment prescribed by the vendor of the BWTS.

During ballast water tests samples will be taken sequentially, covering the entire intake or discharge periods.

4.3 Abiotic quality

The land-based test cycles have to be carried out at specific water qualities as defined in the G8-guidelines. The NIOZ-harbour represents a brackish water environment with a salinity varying between 20 and 35 PSU. High salinity water originating from the North Sea is taken in around high tide. Low salinity water from the Wadden Sea is taken in around low tide. The salinity of the Wadden Sea water depends on the discharge of freshwater from Lake IJssel, which itself depends on the amount of rainfall and on the flow rates in the rivers Rhine and IJssel. In an effort to maintain a minimum 10 PSU salinity difference as requested under § 2.3.17 of G8, per tank 17 m^3 freshwater is added in the pipelines to the natural water prior to the pump to reduce the ambient salinity (ca. -2 PSU) and 8 m^3 brine (100 kg m^{-3} industrial quality salt) is added to increase salinity (ca. + 2 PSU) at the second set of test series. At present only brackish and high salinity seawater conditions can be tested at NIOZ.

In addition, per ballast tank 20 litre (16-18 kg dry weight) of mud from the nearby Mokbaai (Figure 2.1) was added to the low salinity tests in order to reach the required TSS value of >50 mg/L. Although calculations show that this amount of mud should increase TSS in 250

m^3 to 60-70 mg/L, the actual concentration measured in the augmented test water was lower. These lower actual TSS values were due to difficulties in keeping high density particles in suspension and because the filters used for measuring TSS do not retain all particles (see 4.3.2). The organic carbon concentration is important in testing systems that use oxidizing agents as active substances. DOC concentrations are usually below 5 mg/L in low salinity test water but no DOC additions are made because the high POC values (>10 mg/L) in the NIOZ test water are considered to compensate for that. In other words, the total organic load in low salinity test water is sufficiently high.

4.3.1 Salinity, Temperature and pH

Water samples for salinity, temperature and pH are collected in 10 L buckets. Measurements are either done immediately or after storage (maximum 6 hours) in the dark and at ambient temperature. Salinity is measured with a digital conductivity meter. Temperature is measured with a calibrated digital thermometer. pH is measured with a calibrated digital pH meter.

4.3.2 TSS/POC (Total Suspended Solids/Particulate Organic Carbon)

For TSS analysis GF/C filters (Glass Fibre/C) with a pore size of approximately 1.2 µm are used to retain the suspended solids. The GF/C filter is the standard filter at NIOZ for TSS analysis. After filtering a known amount of sample the pre-weighed filters are dried at 60°C for at least 8 hours and weighed again. The concentration of TSS per litre can be calculated from the sample volume and the weight difference of the filter before and after sampling. TSS is expressed in mg/L.

The amount of estimated TSS varies with the type of filter that is used. This became apparent in 2012 when a 'mud balance' was made: the gravimetrically determined amount of mud that was added (see 4.3) was compared to the amount of mud in suspension in the NIOZ installation measured as TSS using GF/C filters. A good balance could only be made when a considerable part of the mud added was not included in the TSS measured.

The standard GF/C filter is rather course and will not retain particles that are smaller than 1 µm. An alternative would be the GF/F filter that has a pore size of approximately 0.7 µm. The use of different filter types was investigated at NIOZ in 2012 using water with suspended Mokbaai mud. As expected, the GF/F filters retained more suspended solids, on average 25 ± 17% mg/L. In other words, the use of GF/C may severely underestimate the true TSS concentration.

To determine the POC concentration the GF/C filter is combusted overnight at 500°C and allowed to cool in a dessicator and weighed again. The POC is calculated from the weight difference between this measurement and the dry TSS weight. POC is expressed as mg C/L.

4.3.3 Dissolved Oxygen

Fixed samples in Winkler bottles are acidified with H_2SO_4 prior to measuring the optical density (OD) at 456 nm with a spectrophotometer. The oxygen concentration is calculated using standards in µM O_2/L or mg O_2/L. Because both salinity and temperature change over the season the oxygen concentrations is expressed as percentage relative to the natural saturation value for the given temperature and salinity.

4.3.4 Dissolved Organic Carbon (DOC)

The DOC concentration is determined in the laboratory by a high temperature combustion method using a Shimadzu TOC-Vcpn analyser according to Reinthaler & Herndl (Reinthaler & Herndl 2005). Standards are prepared with potassium hydrogen phthalate (Nacalao Tesque, Inc, Kioto, Japan). The mean concentration of triplicate injections of each sample (three in total) is calculated. The average analytical precision of the instrument is <3 %.

4.4 Biological quality

In order to establish the biological efficacy of the BWTS it should be tested with water containing a high concentration of organisms as well as a sufficient biodiversity (§ 2.3.20 of G8). This is required by G8 to guarantee the effectiveness of the BWTS in different ecosystems across the globe. The variety of organisms in the influent test water should be documented according to the size classes mentioned in Table 3.3.

For completeness, the plankton fraction <10 µm and the total phytoplankton viability are included in the NIOZ analyses although this is not required by the G8-guideline.

4.4.1 Organisms ≥50 µm

Organism in this size class are concentrated with plankton nets and plankton gauze. They are counted live using a binocular microscope. To establish the minimum dimension of an organism the "body" should be measured, i.e. not antennae, tails etc. Examples are presented in Figure 4.1.

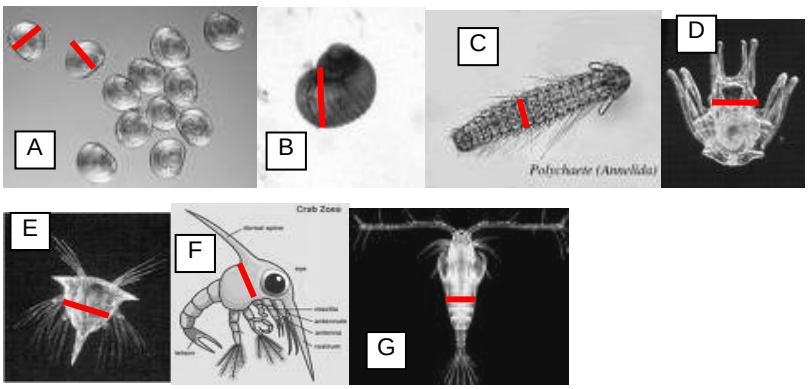

Figure 4.1. Minimum dimension measurements (red line) in selected organism types: A = bivalve larvae, B = gastropod larvae, C = worm, D = echinodermata larvae, E and F = crustacean larvae and G = copepod.

The viability of the organisms is assessed with Neutral Red, which stains living organisms only and does not affect their survival rate. This viability assessment remains unaffected by the possible death of organisms during staining or during sample analysis due to, for instance, warming of the sample. This is because organisms that die after addition of the Neutral Red will still be clearly stained, while those already dead prior to the addition will not be stained.

Neutral Red stains all major plankton groups, including phytoplankton, but it seems to have some practical limitations for bivalve larvae. For the latter movement, including that of hart and gill is used to verify viability. This depends on the expertise of the person analysing the samples. Therefore, only persons with a dedicated training period will analyse samples. Organisms that are able to swim are also considered alive. When in doubt, the organism can be poked with a dissection needle. The procedure is outlined in Figure 4.2.

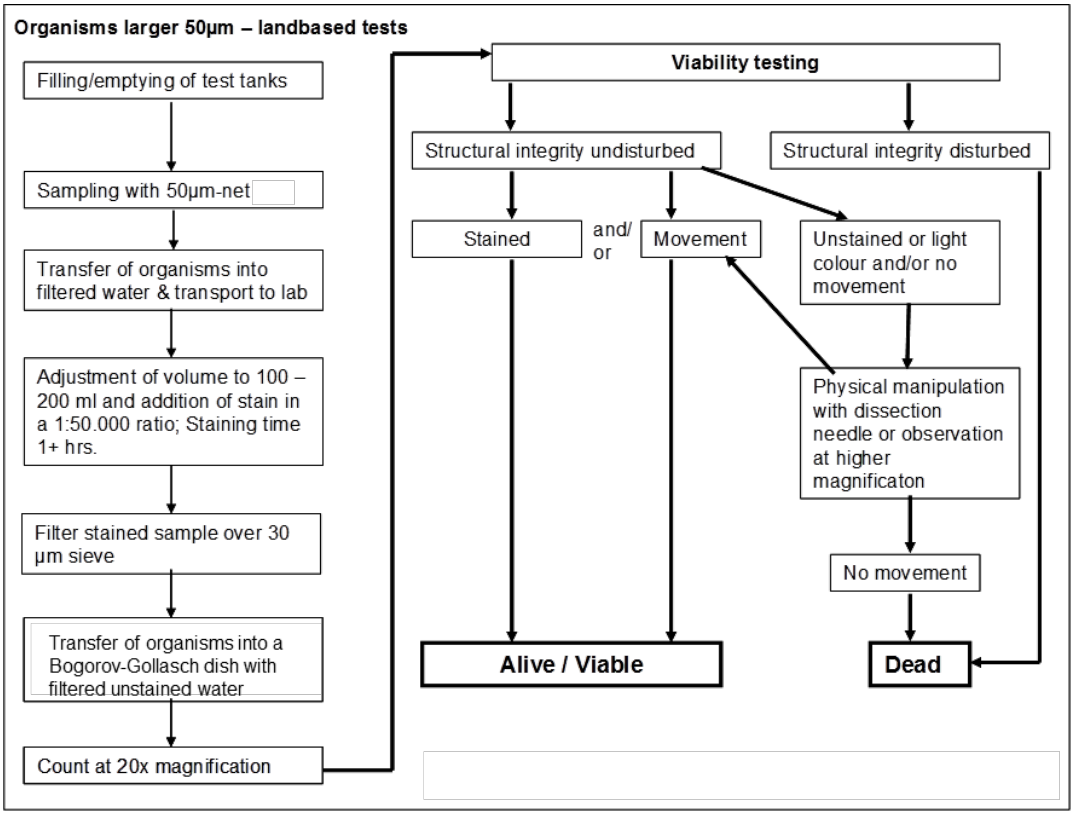

Figure 4.2. Sampling and viability assessment for organisms larger than 50 µm during land-based tests.

4.4.2 Organisms 10≤µm<50: phytoplankton

Organisms in the 10≤µm<50 size class are analyzed by flow cytometry, a semi-automated method used at NIOZ for the counting of phytoplankton, bacteria and viruses. The viability of the organisms present will be addressed by using specific fluorescent dyes methods as explained below.

Samples are counted using standard protocols covering the particles in the size range of ca. 2 - 50 µm. Total analysis time is equal to an exact sampling volume of 1 mL or otherwise when relevant. Of all particles present in the volume counted, the cell size and the presence or absence of chlorophyll-*a* fluorescence will be measured. Only phytoplankton has chlorophyll-*a* fluorescence.

Absolute numbers, cell sizes and chlorophyll-*a* content of the particles is analyzed using the software package FCS Express V3 or V4 (DeNovo, US). Cell sizes are estimated relative to 10 µm standard fluorescent beads (Flow-Check Fluorospheres, Beckman Coulter #660539).

For measuring viable phytoplankton, three subsamples are stained with SYTOX-Green (Veldhuis et al. 2001). This nucleic acid specific dye only stains DNA of cells with a compromised cell membrane, which are then considered dead. Of each phytoplankton cell present the green SYTOX fluorescence will be determined and compared with the green autofluorescent signal (Veldhuis et al. 2001, Cassoti et al. 2005, Peperzak & Brussaard 2011).

4.4.3 Organisms 10≤µm<50: microzooplankton

The samples are analyzed with an inverted microscope at 200x magnification (method by Utermöhl). The fixed samples (or sub-samples thereof) are transferred into settling chambers and neutralized using sodium thiosulfate. After this, the sample is stained using Bengal rose stain. This stain specifically stains organic material and helps to identify

organisms between sediment particles. After staining the samples are left undisturbed in the dark to settle. Live-dead-separation in these samples is mainly based on the structural integrity of organisms. Because the structural integrity of organisms may not be compromised after UV-treatment, incubation experiments were performed in 2012 to investigate if intact microzooplankton was indeed still viable (see § 4.6).

4.4.4 Human pathogens

The samples for human pathogens are taken in special bottles of 300 or 600 mL and send to a contract laboratory (Eurofins/ C.mark) for further analysis. All analyses are carried out according to NEN/ISO standards. Analysis for *Escherichia coli* is carried out according to ISO 9308-3 for the analysis of surface waters. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Analysis for the *Enterococci* group is carried out according NEN/ISO 7899-2. For this the samples are filtered through membrane filters (pore size 0.45 µm) and these filters are incubated on a selective agar plate. Incubation is 44 ± 4 hours at 36 ± 2°C on Slanetz & Bartley medium.

4.4.5 Total heterotrophic bacteria

The classical method for counting bacteria in many applications is based on plating on selective media, where each individual cell is supposed to form a colony after an appropriate incubation time. Unfortunately, for studies in the aquatic environment this approach is by far insufficient for various reasons (Gasol & Del Giorgio 2000). Therefore, the total bacteria concentration in fixed samples is determined by flow cytometry using the DNA-specific stain PicoGreen (Veldhuis et al. 1997, Gasol & Del Giorgio 2000).

The dye PicoGreen is a green nucleic acid specific dye that only stains ds (double stranded) DNA, with little or no cross-over for ss (single stranded) DNA and RNA (Veldhuis et al. 1997). This makes the staining method ideal to for staining of DNA and therefore to determine bacterial abundance. Flow cytometric analysis shows a clear signal with an excellent signal to noise ratio and bacteria are made visible easily and distinguishable from viruses and larger organisms. This approach has extensively been compared with bacteria staining and counting using an epifluorescent microscope, resulting in nearly identical results. However, because the flow cytometer method is much faster (results are obtained within 100 seconds and over 100 samples can be analyzed per day), and highly reproducible this counting method is be preferred above the far more time consuming and labour intensive microscopic observations.

4.5 Additional measurements

NIOZ strives to improve and speed-up existing methods for counting viable organisms. In addition NIOZ performs measurements and (incubation) experiments that are supplementary to the G8-guidelines. The reason for doing this is to be able to better and faster evaluate the performance of the BWTS's tested.

4.5.1 PAM measurement for total phytoplankton viability

The photochemical efficiency of photosystem II is an indicator of the physiological 'health' condition of phytoplankton cells. It is a bulk variable that is measured using a Pulse Amplitude Modulated (PAM) fluorimeter (Schreiber et al. 1993). The simple fluorescence ratio Fv/Fm gives a qualitative indication of the photosynthetic efficiency of the phytoplankton community. In addition, the maximum fluorescence value Fm is an indication of phytoplankton biomass. If no fluorescence peak can be measured, the phytoplankton is considered dead.

4.5.2 Phytoplankton <10 µm

Many species of the phytoplankton community have cells that are smaller than 10 µm. Although not in the D-2-standard, the efficacy of ballast water treatment systems can be measured by counting these small algae. The organisms in this size class are analyzed by flow cytometry, as explained for the 10≤µm<50 phytoplankton size class.

4.6 Incubation experiments

In order to measure the potential regrowth of phytoplankton after the first and second treatment of the Aquarius™-UV system, 10 L samples were incubated in a climate room under favourable conditions. After an incubation time of seven days the 10≤µm<50 and <10 µm phytoplankton concentrations were measured using the methods outlined above. Average growth rates were calculated as:

$$\mu \text{ (divisions per day)} = (\ln(T_7 / T_0)/e)/7 \qquad [1]$$

With T_7 and T_0 as the concentration on day 7 and 0 respectively, and e = Euler's number.

Starting in 2012 the incubation experiments were also used to assess the viability of microzooplankton. In previous years their viability was assessed from the structural integrity or 'intactness' of the organisms (§ 4.4.3). However, especially in the case of UV-treatment, non-vital organisms may appear intact but they might not be viable due to DNA damage. Because the concentrations of microzooplankton is relatively low compared to phytoplankton in the same (10≤µm<50) size range or to the concentrated mesozooplankton (>50 µm) it is not very practical to use a vital stain and microscopy or flow cytometry to count vital microzooplankton cells. Therefore, intermediate salinity test water that was treated by the Aquarius™-system was incubated. In addition to the phytoplankton, the microzooplankton was sampled and the samples were concentrated and counted using the same procedure as for the harbour and BWTS samples (§ 4.4.3).

Figure 4.3. shows that microzooplankton cells in two control samples that were not UV-treated were present at a concentration of 1 to 3 cells mL^{-1} on the day of discharge. After one day incubation their concentrations more than doubled, indicative for the viability of these cells. On the other hand, the corresponding samples that that had been treated by the Aquarius™-UV system, operating at 60% power, contained low concentrations (<0.5 cells mL^{-1}) of intact microzooplankton cells that had completely disappeared after one day of incubation. At the second day of incubation the microzooplankton concentrations were still zero (Figure 4.3) and this remained so until the end of the experiments after one week of incubation. Clearly, the observed intact microzooplankton cells were non-viable after the second treatment by the Aquarius™-UV system operating at 60% design lamp power.

Therefore, a similar treatment at 100% design lamp power, as in 2011, would also have rendered the microzooplankton cells non-viable.

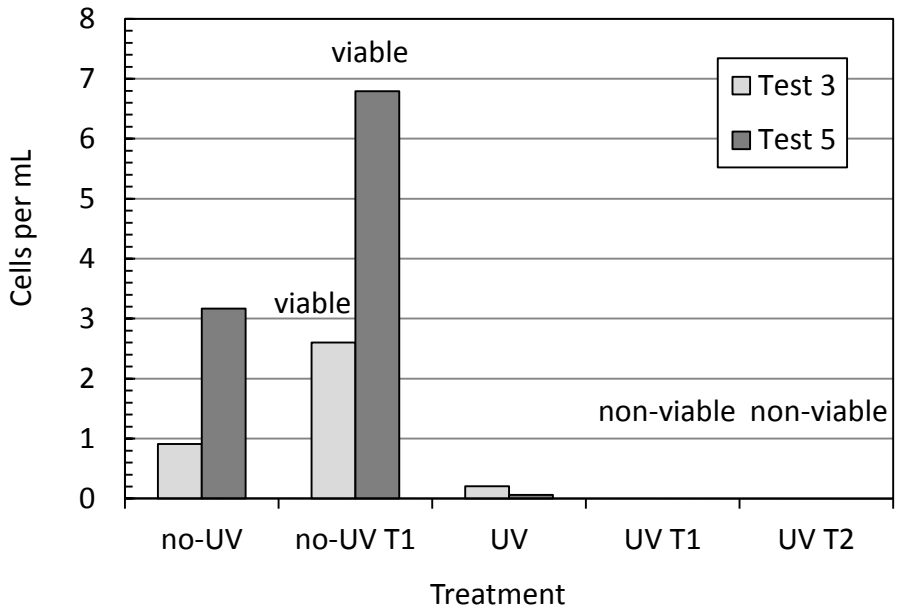

Figure 4.3. Microzooplankton viability tested in two incubation experiments from tests 3 and 5 of 2012. In the control samples (no UV) the microzooplankton concentrations nearly doubled after one day (no UV T1), these cells were viable. On the contrary, low concentrations of intact cells were present in water that had been treated by the Aquarius™-UV system operating at 60% power (UV). These cells completely disappeared after one day (UV T1) and did not reappear (UV T2). Thus, the UV-treated cells were non-viable.

4.7 Data analysis

4.7.1 Confidence intervals

In order to calculate if the differences between treatments are statistically significant t-tests were performed by calculating the variables' averages, the standard deviations (sd) and by applying the correct t-value for a given degrees of freedom, that is the number of observations (n) minus 1, from a t-table. The 95% confidence interval was calculated as:

$$95\%\ \text{c.i.} = t_{df,\ 95\%} \times sd / n^{0.5} \qquad [2]$$

Averages with ± 95% confidence intervals that do not overlap are significantly different.

4.7.2 Efficacies

Efficacy (E) is calculated from the logarithmic reduction in organism concentration (C, as number of organisms per volume) before and after treatment:

$$E = \log_{10} (C_{\text{before treatment}} / C_{\text{after treatment}} + 1) \qquad [3]$$

To the "after treatment" concentration 1 is added to prevent division by zero.

For organisms >50 µm the minimum efficacy according to G8 and D2 is:

$$E_{>50\ \mu m} = \log_{10} (100{,}000 / 9 + 1) = 4.0 \qquad [4]$$

For organisms 10≤ µm<50 µm the minimum efficacy according to G8 and D2 is:

$$E_{10\leq\ \mu m<50\ \mu m} = \log_{10} (1{,}000 / 9 + 1) = 2.0 \qquad [5]$$

For the 10-50 µm organisms the concentration after the pump (but before treatment) was used in calculating efficacy. This group of organisms contain chain-forming phytoplankton that should be measured as solitary cells (§3.1.1 G2). The ballast pump destroyed the chains but not the cells, giving the required estimate.

4.7.3 Multivariate statistical tests

To summarise the overall effect of the ballast water treatment, non-parametric tests were performed in PRIMER version 6.1.13. An example of such a test is given in chapter 3. The controls were the untreated test waters after a five day holding period. Each control was compared to the corresponding treatment tests that were also sampled on day 5, after the second UV-treatment.

For the abiotic comparison, the variables were: temperature, pH, oxygen saturation, TSS, POC and DOC. The values were normalised and the resemblance measure used was Euclidean distance. Biotic variables were the concentrations of total phytoplankton (10≤ µm<50), viable microzooplankton (10≤ µm<50) and viable mesozooplankton (>50 µm). The data of the pathogenic bacteria was not included because their concentrations were below detection limits. The biotic variables were fourth root (>50 µm) or square root transformed (other size classes) and the resemblance measure used was Bray-Curtis.

The difference between controls and treated water was visualised in a non-parametric multi-dimensional scaling (NMS) diagram after a SIMPROF (similarity profile) test in Cluster Analysis in order to distinguish groups of data. If relevant, the similarity profile was used to draw a line around samples with a certain percentage similarity in the NMS diagram. The null-hypothesis that controls and treatments were not different was tested with a one-way ANOSIM (analysis of similarities, 9999 permutations).

5 D-2 AND G8 RESULTS AND DISCUSSION

In this chapter all data obtained relevant to the D-2 requirements and G8-guidelines measured during the testing of the Aquarius™-UV system in 2011 and 2012 are presented and discussed. The intermediate salinity tests were performed from April to May 2011, the high salinity tests in June 2011.

5.1 Abiotic quality

Table 5.1. Abiotic quality of the NIOZ test water. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Avg ± c.i. is the average ± 95% confidence intervals. The range is the minimum and maximum value. Salinity in psu. TSS, POC and DOC are in mg/L.

	Set I		set II		set III	
	avg ± c.i.	range	avg ± c.i.	range	avg ± c.i.	range
Salinity	28 ± 2	25-30	25 ± 2	23-27	36 ± 2	34-38
TSS	41 ± 13	26-52	46 ± 9	38-55	11 ± 3	8-19
POC	13 ± 4	9-18	12 ±2	9-14	5 ± 1	4-6
DOC	2.2 ± 0.2	2.0-2.6	2.5 ± 0.4	2.0-2.9	2.2 ± 0.2	2.0-2.2

In general the basic requirements for testing were met. The extremely low freshwater discharge into the Wadden Sea in spring 2011 led to relatively high salinities in the intermediate salinity range at 100% UV. Spring 2012 was much wetter and the average salinity in the intermediate salinity range was therefore lower than in 2011 and, on average, 11 PSU lower than the high salinity tests (Table 5.1). The total range of salinities during the tests ranged from 25 to 38 PSU.

Furthermore, it should be noted that within the salinity ranges tested the efficacy of the UV-system is not influenced by the salinity. In 2011 the correlation between the percentage UV-transmission in a 10 mm quartz cuvette (UV-T10) of Lake IJssel freshwater diluted with oceanic saline water was:

$$\text{UV-T10 (\%)} = 75 + 0.57 * \text{Salinity} \qquad r^2 = 0.96 \qquad [3]$$

Using equation [3] the UV-T10 of water with salinities of 25 and 35 PSU is calculated to be 89 and 95%. A further reduction of the salinity to e.g. 20 PSU would have decreased T10 from 89 to 86% which is not convincingly lower, i.e. it would not have made testing more difficult. In other words, for testing UV-BWT systems at NIOZ, the actual salinities are of minor importance.

The TSS concentrations in the intermediate salinity ranges were not significantly different from 50 mg/L. However, despite the addition of mud to the intake water, the ranges of TSS values were relatively low. These values are low estimates of the actual TSS concentrations because if GF/F instead of the GF/C filters were used to retain the suspended solids (see 4.3.2), TSS would have been 25% higher: on average >50 mg/L.

It is generally expected that high TSS values negatively influence UV-T. Although this is true in principle, UV-T measurements by NIOZ during filter experiments in 2011 (not related to the present Aquarius™-UV test) showed that in the range of 0 to 100 mg TSS/L an increase of 10 mg TSS/L lead to a reduction of UV-T10 of only 1%. Therefore, it is not expected that even twice the TSS concentrations as those achieved during the tests in 2011 would have reduced the efficacy of the Aquarius™-UV system.

In addition, low DOC concentrations were measured in the intermediate salinity range. This was no surprise because DOC concentrations usually are below 5 mg/L in low salinity test

waters in the NIOZ harbour. No effort to increase DOC were made because the high POC values (>10 mg/L) in the test water are considered to compensate for the low DOC values. In other words, the total organic load in intermediate salinity range was sufficiently high, although in a slightly different manner as described in G8.

It is concluded that the abiotic quality of the naturally available test water met the requirements for conclusive testing of the Aquarius™-UV system.

5.2 Environmental variables

The results of all abiotic variables that were measured during the tests of the Aquarius™-UV system are presented in Table 5.2.

Table 5.2. Environmental variables in NIOZ test water. Samples from control and treated water at intake (T0) and discharge (T5). The numbers are averages ± 95% confidence interval; non-overlapping intervals indicate a significant difference between two averages. Oxygen saturation levels were calculated for the corresponding temperatures and salinities of the samples. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power.

set I	control		treated		
	T0	T5	T0	T5	unit
Temperature	13 ± 4	13 ± 3	13 ± 3	13 ± 3	°C
pH	8.5 ± 0.2	8.4 ± 0.2	8.4 ± 0.2	8.4 ± 0.2	-
Dissolved Oxygen	111 ± 7	75 ± 23	111 ± 9	86 ± 26	%
TSS	41 ± 13	10 ± 3	29 ± 9	11 ± 4	mg/L
POC	13 ± 4	5 ± 1	10 ± 3	6 ± 2	mg/L
DOC	2.4 ± 0.2	2.5 ± 0.6	2.5 ± 0.3	2.4 ± 0.2	mg/L

set II	control		treated		
	T0	T5	T0	T5	unit
Temperature	9 ± 2	10 ± 2	9 ± 2	10 ± 2	°C
pH	8.4 ± 0.1	8.3 ± 0.1	8.4 ± 0.1	8.3 ± 0.1	-
Dissolved Oxygen	113 ± 5	98 ± 7	114 ± 6	99 ± 5	%
TSS	46 ± 9	11 ± 1	37 ± 8	12 ± 3	mg/L
POC	12 ± 2	6 ± 1	10 ± 1	6 ± 1	mg/L
DOC	2.5 ± 0.4	2.4 ± 0.3	2.4 ± 0.3	2.4 ± 0.2	mg/L

set III	control		treated		
	T0	T5	T0	T5	unit
Temperature	17 ± 1	17 ± 0	17 ± 1	17 ± 0	°C
pH	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.2	8.3 ± 0.0	-
Dissolved Oxygen	101 ± 8	84 ± 6	102 ± 7	82 ± 17	%
TSS	11 ± 3	10 ± 5	12 ± 5	7 ± 1	mg/L
POC	5 ± 1	5 ± 1	5 ± 1	4 ± 0	mg/L
DOC	2.2 ± 0.2	2.1 ± 0.3	2.1 ± 0.1	2.1 ± 0.2	mg/L

Judging from the individual variables (Table 5.2) there were no significant changes between the control water and the treated water on the day of intake (T0), although TSS values were considerably reduced at the intermediate salinities after the first treatment that included filtration over a 40 µm mesh screen. The reduction in TSS by filtration also led to reduced POC concentrations.

On T5, at discharge, TSS in both treated and control water at intermediate salinities were significantly lower than at T0, presumably by sedimentation in all ballast water tanks during the five day holding period. A comparable reduction between intake at T0 and discharge at T5 was seen in POC at intermediate salinity. At high salinity the differences in TSS and POC between intake and discharge were not significant (Table 5.2).

A clear and significant difference between intake and discharge waters was found for the dissolved oxygen concentrations. This is not related to the treatment itself, because there was no significant difference with the control water. The reduction of oxygen saturation in both control and treatment tanks is related to heterotrophic activity. Negative effects of low oxygen levels are expected below 10% saturation (Peperzak & Poelman 2008) but such low values were never reached in the discharge waters.

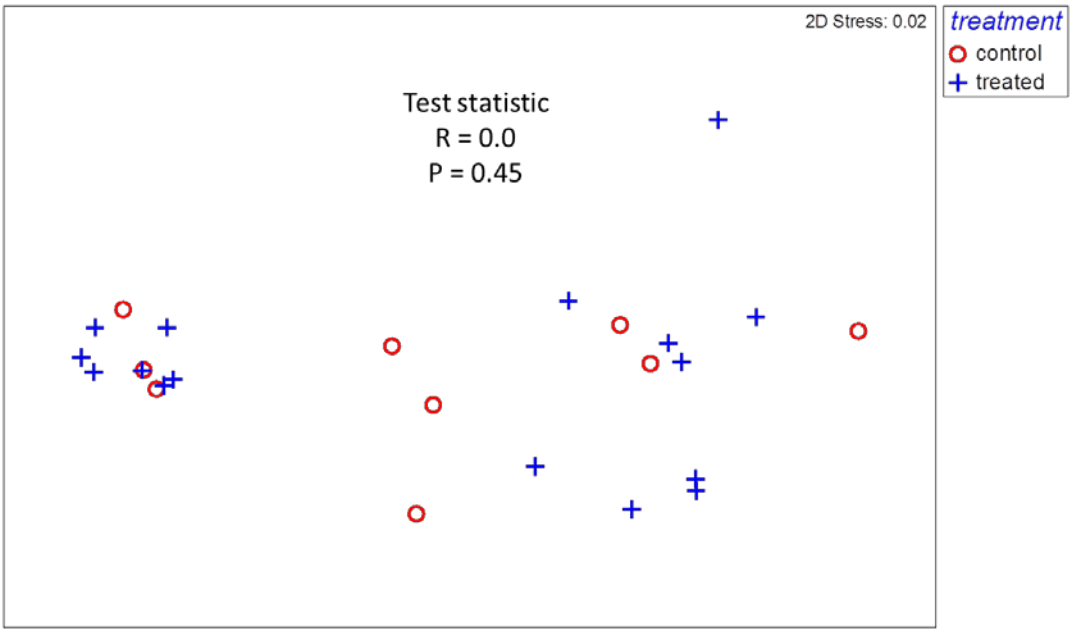

Figure 5.1. Diagram of the mathematical distances between control and treatment samples at T5 calculated from temperature, pH, oxygen saturation, TSS, POC and DOC. The positions of control and treatment samples overlap, indicating that there is no clear difference between the two groups of samples. This is corroborated by the low value of the test statistic R.

The overall effect of the ballast water treatment on the abiotic variables is visualised in Figure 5.1: there was no clear difference between control and treatment samples. The test statistic R was zero and, as to be expected, the difference was not significant (P>0.05). In other words, the null hypothesis that there is no difference between control and treatment could not be rejected.

In summary, treatment with the Aquarius™-UV system did not significantly change the abiotic quality of the discharge water. TSS and POC concentrations were reduced. Oxygen saturation levels were lowered but remained high enough to prevent local hypoxic conditions.

5.3 Overall biological quality

Table 5.3. Organism concentrations in NIOZ test water at the intermediate and high salinity test regimes. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Cfu/100 mL is colony forming units per 100 mL.

set I			
organisms	average	range	unit
≥ 50 µm	101	50-148	x1000 per m3
10 ≤ µm < 50	2.3	1.7-3.0	x1000 per mL
heterotrophic bacteria	470	124-762	x1000 per mL
E. coli	<10	<10	cfu/100 mL
Enterococci	<1	<1	cfu/100 mL

set II			
organisms	average	range	unit
≥ 50 µm	107	50-130	x1000 per m3
10 ≤ µm < 50	1.8	1.0-2.7	x1000 per mL
heterotrophic bacteria	373	124-563	x1000 per mL
E. coli	<10	<10	cfu/100 mL
Enterococci	<1	<1	cfu/100 mL

set III			
organisms	average	range	unit
≥ 50 µm	58	32-88	x1000 per m3
10 ≤ µm < 50	0.6	0.5-0.9	x1000 per mL
heterotrophic bacteria	350	339-361	x1000 per mL
E. coli	<10	<10	cfu/100 mL
Enterococci	<1	<1	cfu/100 mL

In general the G8-requirements were met in most of the tests (Table 5.3). On average the concentrations of organisms at intermediate salinities were above, while at high salinity they were slightly below the requirements. These relatively low values are probably due to the aberrant weather in the spring of 2011 which was sunny with very low rainfall and freshwater discharge into the sea, resulting in lower plankton stocks in late spring-early summer. On the other hand, the biodiversity of the test water was extremely high. The test water contained a total of 17 different phyla in the 10-50 and >50 µm size classes. The total number of species in each G8-size class is 59 (10 phyla) for the 10≤µm<50, and 40 (12 phyla) for the ≥50µm size classes (Table 5.4). Many organisms that were observed in the test water could not be identified to the species level (Appendix 1). Instead they were classified in a higher taxonomic group (see the first note of Table 5.4 for the taxonomic system). This means that the true number of species was even higher.

In conclusion, the requirements for biological quality of the test water during the test of the Aquarius™-UV system were met. A shortage of numbers in the high salinity tests was compensated by a high biodiversity in all tests.

Table 5.4. Biodiversity as number of phyla and species in NIOZ test water. Classification according to phylum and to size class based on data from the 2011 and 2012 spring and early summer test season. Organisms <10 µm that are not bacteria, are not part of the D-2 regulation.

Phylum *	<10 µm	10-50 µm	>50 µm
Amoebozoa		1	
Alveolata **			
Annelida			5
Arthropoda		1	13
Bryozoa			1
Cercozoa		1	
Chlorophyta ***	4		
Choanozoa	1		
Chromista	3		
Ciliophora	7	20	9
Cnidaria			2
Cryptophyta	1	1	
Ctenophora			1
Cyanobacteria	1		
Echinodermata			1
Euglenozoa		1	
Haptophyta	4		
Mollusca			4
Myzozoa	2	14	2
Nematoda			1
Ochrophyta	22	28	7
Protozoa incertae sedis		1	
Rotifera			1
unknown	5	1	
Total number of phyla	10	10	12
Total number of species/species groups	49	59	40

* The taxonomic system is as follows: Kingdom (Archeae, Bacteria, Animalia, Chromista ("Algae"), Plantae) – Subkingdom – Infrakingdom – Phylum – Subphylum -Division – Class – Subclass –Superorder – Order – Family – Genus – Species

** Division (no phylum for this group)

*** The phylum 'unknown' contains several species of unidentified phytoplankton flagellates

5.3.1 Organisms ≥50 µm

The most abundant organisms in the ≥50 µm size class are zooplankton. They were concentrated with plankton nets and counted with a binocular microscope.

Table 5.5. Concentrations of viable organisms ≥50 µm per m^3. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Efficacy is the logarithmic reduction of organisms in treated water on discharge (T5) compared to test water. *based on five tests

Set I						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	147,750	70,550	94,000	28	1	4.9
2	147,750	70,550	94,000	37	1	4.9
3	80,400	38,400	38,500	52	4	4.2
4	80,400	38,400	38,500	139	1	4.6
5	50,350	33,850	33,950	67	1	4.4
6	98,050	47,300	47,700	20	6	4.1
average	100,800	49,850	57,800	57	2	4.5

Set II						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	117,900	40,350	38,050	54	5	4.3
2	117,900	40,350	38,050	69	2	4.6
3	50,350	33,850	33,950	137	0	4.7
4	98,050	47,300	47,700	29	13	3.8
5	129,500	53,500	43,400	32	4	4.4
6	129,500	53,500	43,400	13	4	4.4
average	107,200	44,800	40,800	56	3*	4.5*

Set III						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	87,750	41,900	74,250	467	0	4.9
2	87,750	41,900	74,250	163	0	4.9
3	39,900	19,050	11,400	255	0	4.6
4	39,900	19,050	11,400	193	1	4.3
5	32,150	15,350	12,900	22	1	4.2
average	57,500	27,450	36,840	220	0	4.6

On average, the concentration of >50 µm organisms in the test water of the intermediate salinity tests was more than the 100,000 required by G8 but for the high salinity test this was less.

After a 5 day holding period the number of organisms in the control tank was hardly changed compared to the concentrations after intake on day 0. This means that the

organisms that were tested were still in a good condition. In all tests the minimum concentration of 10 x D-2 (100 m^{-3}) was easily met.

The first treatment with the Aquarius™-UV system, 40 µm filtration and UV radiation, reduced the concentrations of organisms ≥50 µm by a factor 100-1000x.

After the second UV-treatment at discharge the concentrations of viable organisms ≥50 µm were reduced to values well below the D-2-standard, except in test 4 (intermediate salinity, UV-power 60%) where 13 organisms per m3 were counted. Some of these organisms were large and intact copepods that were not expected to pass the 40µm filter. Based on this observation the filter was checked and a seal was found to be leaking under pressure. After replacing this seal tests 5 and 6 were performed with results compliant with D-2. In effect, the number of passed tests (five) was one more than required by ILT for the 60% UV-power test cycle. Furthermore, six tests were performed and passed at the intermediate salinity 60% UV-power test cycle. These extra tests provide sufficient confidence that the Aquarius™-UV system is capable of good performance in land-based tests under the most difficult, intermediate salinity, circumstances.

The efficacies at the intermediate salinity tests were 4.5 which exceeds the required 4.0 by a factor of 3 on a linear scale. In other words, the test result is not 9 (<10 per m-3) but three individual organisms on average (Table 5.5).

Although the numbers of >50 µm organisms in the high salinity test were lower as suggested by G8, in three out of five tests the number of surviving organisms was less than the detection limit (<0 per m3). In addition, the efficacy of the treatment (E = 4.6) exceeds the required 4.0 and is even slightly higher than the efficacies obtained at the intermediate salinity tests.

The data from the testing of Aquarius™-UV system strongly demonstrates that following the second UV treatment the concentration of organisms ≥50µm meets the level stipulated in the D-2 standard. These test results clearly demonstrate the efficacy of the treatment to deliver compliant results.

5.3.2 Organisms 10≤µm<50: phytoplankton

The most abundant organisms in the 10≤µm<50 size class are phytoplankton. Counts performed by flow cytometry are presented in Table 5.6, and additional fluorimetric vitality measurements in §5.4.1. Microzooplankton organisms that fall in the same size category are presented in the paragraph 5.3.3.

Table 5.6. Concentrations of 10≤µm<50 phytoplankton per mL. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Efficacy is the logarithmic reduction of viable organisms in treated water on discharge (T5) compared to the total phytoplankton concentration in the test water.

set I						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	1,512	2,996	116	2,502	0	3.5
2	1,512	2,996	116	1,270	0	3.5
3	916	1,740	100	685	0	3.1
4	916	1,740	100	326	0	3.2
5	268	1,883	107	1,202	0	3.3
6	657	2,662	131	3,241	0	3.4
average	964	2,336	112	1,538	0	3.3

set II						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	1,578	2,229	11	2,116	6	2.5
2	1,578	2,229	11	2,156	4	2.6
3	268	1,883	107	2,094	3	2.7
4	657	2,662	131	1,172	0	3.4
5	892	986	124	969	6	2.1
6	892	986	124	1,572	2	2.5
average	978	1,829	85	1,680	4	2.7

set III						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	725	504	100	460	0	2.7
2	725	504	100	344	0	2.7
3	646	484	94	951	0	2.4
4	646	484	94	386	1	2.6
5	831	874	94	465	*	
average	715	570	96	521	0	2.6

*not measured

On average the concentration of 10≤µm<50 organisms in the test water of the intermediate salinity tests was more than required by G8. The increase in cell numbers from test water to the control at T0 is caused by the ballast water pump. The turbulence caused by this pump breaks up colonies of diatoms that are too large to count accurately with the flow cytometer. Once these colonies are disrupted, the still viable cells achieve a size that can be easily measured by the flow cytometer and the cell concentrations apparently increase. In early

summer, when the high salinity tests were performed, the number of colony-forming diatoms is much lower and the effect does not occur. Unfortunately, the phytoplankton concentrations in early summer 2011 were slightly below the required 1000 per mL. However, it should be noted that the phytoplankton concentrations are a minimum value for the organisms in this size class because the microzooplankton concentrations need to be added to the total of 10≤µm<50 organisms. In spring 2012, when microzooplankton counts were made consistently, their concentration ranged from <10 to 140 per mL indicating that they may contribute a considerable fraction of the abundance of 10≤µm<50 organisms.

After a five day holding period the number of organisms in the control tank was reduced compared to the concentrations after intake on day 0. This is caused by predation by the zooplankton that is active in the dark, while the phytoplankton cannot divide under these circumstances. Again, the microzooplankton concentrations need to be added to the total of 10≤µm<50 organisms. On average, the minimum concentration of 10 x D-2 (100 per mL) was met.

At discharge, the ballast water had undergone two treatments. The first treatment with the Aquarius™-UV system, 40 µm filtration and UV, hardly reduced the concentrations of 10≤µm<50 phytoplankton. Two explanations can be given. First, the majority of organisms in this size range are much smaller than 50 µm and may well pass the 40 µm filter. Second, UV does not instantaneously destroy cells, so they still will be counted in samples taken immediately after treatment. Incubation experiments at NIOZ during previous G8-tests have conclusively shown that a reduction in the order of 100x in phytoplankton cell counts takes places during five days following a first UV-treatment (Stehouwer et al. 2010, Liebich et al. 2012). A comparable reduction in total cell concentrations, i.e. an overestimation of the number of viable cells has been measured in incubation experiments following a second UV-treatment (Stehouwer et al. 2010, Liebich et al. 2012). This overestimation of viable cells was also obvious in the Aquarius™-UV G8-test. The average cell concentrations after the second UV-treatment ranged from 40 to 80 (results not shown) while the number of viable cells is always <1 per mL (Table 5.6).

Six tests were performed and passed for phytoplankton counts in the intermediate salinity test cycles at 60% and 100% UV-power. This number of passed tests was one more than required by ILT for the 60% UV-power test cycle and one more at 100% UV-power than required for G8. Test 4 at intermediate salinity and 60% UV-power failed for organisms >50 µm but passed for the phytoplankton counts. Passing six tests under the most difficult, intermediate salinity, circumstances provides good confidence in the performance of the Aquarius™-UV system in the land-based tests.

The efficacies at the intermediate salinity tests were 2.7 to 3.3 which exceeds the required 2.0 by a factor of 10 on a linear scale. In other words, the test result is not 9 (<10 per mL) but two on average (Table 5.6). Although the performance of the Aquarius™-UV system at 60% was still more than sufficient, the increase in power to 100% showed an even better result.

Although the numbers of 10≤µm<50 phytoplankton organisms in the high salinity test were lower as suggested by G8, in three out of five tests the number of surviving organisms was less than the detection limit (<0 per mL). In addition, the efficacy of the treatment (E = 2.6) exceeds the required 2.0.

In all three test cycles the efficacy of the treatment on phytoplankton was corroborated by independent viability assessments, by PAM fluorimetry and by measuring growth rates in incubation experiments.

The data from the testing of Aquarius™-UV system strongly demonstrates that following the second UV treatment the concentration of 10≤µm<50 phytoplankton organisms meets and exceeds the level stipulated in D-2 standard. These test results clearly demonstrate the efficacy of the treatment to deliver compliant results.

5.3.3 Organisms 10≤µm<50: microzooplankton

The concentrations of microzooplankton are usually a factor of 100 lower than those of the phytoplankton in this 10≤µm<50 size range. Therefore, microzooplankton counts were only made in Aquarius™-UV-treated water and in the control water on the day of discharge (T5).

Microzooplankton was microscopically counted as structurally intact cells. In 2012 incubation experiments showed that that intact cells at T5 are not viable, see chapter 4.6. This means that tests 1 and 2 at intermediate salinity and 100% UV-power are in fact compliant with D2 (Table 5.7).

Table 5.7. Concentrations of 10≤µm<50 microzooplankton per mL for all tests at the day of discharge (T5). The organisms counted were intact cells that were not assessed for vitality or viability. The actual concentrations of viable cells is <1 per mL. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power.

set I		
	Control	Treated
Test	T5	T5
1	7.7	15.7
2	7.7	15.0
3	11.0	8.0
4	11.0	5.0
5	0.9	0.1
6	4.5	0.0
average	7.1	7.3

set II		
	Control	Treated
Test	T5	T5
1	6.9	0.0
2	6.9	0.0
3	0.9	0.2
4	4.5	0.0
5	3.2	0.1
6	3.2	0.0
average	4.3	0.1

set III		
	Control	Treated
Test	T5	T5
1	2.0	0.7
2	2.0	0.3
3	0.2	0.1
4	0.2	0.1
5	1.4	0.1
average	1.2	0.3

In the high salinity tests at 100% UV-power and the intermediate salinity tests at 60% UV-power the numbers of morphologically intact microzooplankton are well below 10 organisms per mL (Table 5.7). In the intermediate salinity range two tests had cell numbers >10 per mL. As for the phytoplankton, UV does not instantaneously destroys microzooplankton cells, so in samples taken directly after treatment an overestimation of viable cells may occur.

Phyto- and mesozooplankton are examined with viability-stains, but the viability of microzooplankton is judged by its structural integrity. UV-radiation can instantly kill organisms (measured by vitality stains) but the loss of structural integrity, followed by a decrease in the concentration of the organisms, may take up to five days (Stehouwer et al. 2010, Liebich et al. 2012). In other words, the method of assessing the viability of microzooplankton has most probably led to an overestimation of their abundance.

In chapter 4.6 it was shown that in 2012 after treatment by the Aquarius™-UV system operating at 60% power the observed intact microzooplankton cells were non-viable. A similar treatment at 100% power as in 2011 would also have rendered the microzooplankton cells non-viable. Therefore, NIOZ is confident that the actual concentrations of all viable organisms, phyto- and microzooplankton in the 10≤µm<50 size range in all tests did comply with the D-2-standard.

It is concluded that all 10≤µm<50 organisms, i.e. the sum of the viable phytoplankton and microzooplankton concentrations were reduced to levels well below those stipulated in the D-2 standard for treated ballast water discharges. This demonstrates the high level of treatment efficacy of the Aquarius™-UV system.

5.3.4 Human pathogens

Table 5.8. Concentrations of *E. coli* and Enterococci (pathogenic bacteria) in colony forming units per 100 mL for all tests. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power.

set I						
	E. coli			Enterococci		
		Control	Treated		Control	Treated
Test	Test water	T5	T5	Test water	T5	T5
1	<10	<10	<10	<1	<1	<1
2	<10	<10	<10	<1	<1	<1
3	*	<10	<10	*	<1	<1
4	*	<10	<10	*	<1	<1
5	<10	<10	<10	<1	<1	<1
6	<10	<10	<10	<1	<1	<1
average	<10	<10	<10	<1	<1	<1

set II						
	E. coli			Enterococci		
		Control	Treated		Control	Treated
Test	Test water	T5	T5	Test water	T5	T5
1	<10	<10	<10	<1	<1	<1
2	<10	<10	<10	<1	<1	<1
3	<10	<10	<10	<1	<1	<1
4	<10	<10	<10	<1	<1	<1
5	<10	<10	<10	<1	<1	<1
6	<10	<10	<10	<1	<1	<1
average	<10	<10	<10	<1	<1	<1

set III						
	E. coli			Enterococci		
		Control	Treated		Control	Treated
Test	Test water	T5	T5	Test water	T5	T5
1	<10	<10	<10	<1	<1	<1
2	<10	<10	<10	<1	<1	<1
3	<10	<10	<10	<1	<1	<1
4	<10	<10	<10	<1	<1	<1
5	*	<10	<10	*	<1	<1
average	<10	<10	<10	<1	<1	<1

*not measured

The Wadden Sea in general and the NIOZ harbour in particular are areas with little or no human waste discharge. As expected, the concentrations of E. coli and intestinal enterococci were very low (Table 5.8). Therefore, no effects of the treatment system were apparent.

All samples tested for E. coli fulfilled the D-2-standard of <250 cfu/100 mL.
All samples tested for enterococci fulfilled the D-2-standard of <100 cfu/100 mL.

5.3.5 Total heterotrophic bacteria

The only regulation applicable to heterotrophic bacteria is a minimum concentration of 10^4 per mL, in the test water at intake. They should also be measured at discharge. No other regulations or guidelines are applicable to the heterotrophic bacteria. The data are presented in Table 5.9.

Table 5.9. Concentrations of total heterotrophic bacteria for all tests at intake and discharge. Concentrations in cells per mL. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. * not measured.

set I		
	Control	Treated
Test	Test water	T5
1	503,000	199,600
2	503,000	277,400
3	762,400	104,200
4	762,400	56,000
5	162,100	74,500
6	124,200	118,700
average	469,500	138,400

set II		
	Control	Treated
Test	Test water	T5
1	412,100	186,200
2	412,100	153,600
3	162,100	113,700
4	124,200	93,200
5	562,900	66,300
6	562,900	254,800
average	372,700	144,600

set III		
	Control	Treated
Test	Test water	T5
1	361,100	43,800
2	361,100	91,300
3	339,100	78,200
4	339,100	65,600
5	*	104,000
average	350,100	76,580

On average the concentration of total heterotrophic bacteria in the test water of all tests was more than a factor of 10 higher than required by G8 (10,000 per mL). After the second treatment at T5 the numbers of total heterotrophic bacteria had declined to 30 - 40%. Conclusions about these differences between T0 and T5 are difficult to make because of the two UV-treatments in between on the one hand, and the capability of these micro-organisms of rapid regrowth on the other.

The concentrations of heterotrophic bacteria were well above the G8-guideline of 10^4 per mL for test water at intake.

5.4 Additional measurements

5.4.1 PAM measurements for total phytoplankton viability

The physiological 'health' condition of the total phytoplankton community is rapidly assessed with the Pulse Amplitude Modulated (PAM) fluorimeter. Phytoplankton cells of all sizes are included in the measurement. If this instrument does not display a fluorescence peak, than the phytoplankton can be considered dead, irrespective of size.

Table 5.10. Physiological status of phytoplankton organisms of all sizes indicated by the Fv/Fm ratio. A low Fv/Fm ratio indicates a low viability. In addition, healthy phytoplankton show a distinct peak during measurement that is undetectable in dead phytoplankton as indicated by different colours. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. *based on five tests

set I					
		Control		Treated	
Test	Test water	T0	T5	T0	T5
1	0.59	0.56	0.16	0.01	0.00
2	0.59	0.56	0.16	0.01	0.01
3	0.46	0.47	0.11	0.02	0.03
4	0.46	0.47	0.11	0.02	0.03
5	0.60	0.59	0.39	0.10	0.07
6	0.58	0.70	0.26	0.03	0.02
average	0.55	0.56	0.20	0.03	0.02

set II					
		Control		Treated	
Test	Test water	T0	T5	T0	T5
1	0.52	0.64	0.25	0.34	0.03
2	0.52	0.64	0.25	0.34	0.05
3	0.60	0.59	0.39	0.30	0.10
4	0.58	0.70	0.26	0.22	0.07
5	0.63	0.61	0.23	0.17	0.02
6	0.63	0.61	0.23	0.17	0.01
average	0.58	0.63	0.27	0.26	0.04*

set III					
		Control		Treated	
Test	Test water	T0	T5	T0	T5
1	0.62	0.59	0.36	0.01	0.01
2	0.62	0.59	0.36	0.01	0.06
3	0.56	0.60	0.18	0.11	0.02
4	0.56	0.60	0.18	0.03	0.03
5	0.59	0.60	0.14	0.01	0.02
average	0.59	0.59	0.24	0.04	0.03

signal: no some normal

The physiology of the total phytoplankton community was high (Fv/Fm > 0.5) in the test water and in the controls at T0 and declined to low values after five days incubation (Fv/Fm < 0.3). After the first treatment the physiological status of the phytoplankton was very low (Fv/Fm < 0.1) in the 100% UV treatments but not in the 60% treatment (Table 5.10). However, in all tests after the second treatment Fv/Fm values were very low and with one exception no signal was detected. This exception took place in test 4 at the intermediate salinity range at 60% UV-power. Interestingly, this was the test that failed on a slightly too high >50 µm organism concentration due to a leaking seal in the filtration unit. Apparently, the PAM fluorimeter picked up a signal of viable phytoplankton cells although the overall (average) Fv/Fm was very low. This viable phytoplankton signal in test 4 was not caused by small phytoplankton cells because the number of viable cells in test 4 was also zero (Table 5.9).

The absence of total phytoplankton viability at discharge concurs with the very low (<1 per mL) concentration of viable cells in the 10-50 µm size range. This fluorimetric measurement may be a fast method to test compliance with the D-2-standard for 10-50 µm phytoplankton, provided a relatively low <10 µm phytoplankton concentration.

5.4.2 Phytoplankton <10 µm

Many algal species have cells that are smaller than 10 µm. This group of small phytoplankton is not mentioned in the D-2-standard. This should certainly be considered as an omission since this size class in particular includes numerous phytoplankton species that are capable of forming Harmful Algal Blooms (HABs) (Liebich et al. 2012). As for larger organisms, the efficacy of a BWTS for these algae can be measured by counting the cells before and after treatment.

Table 5.11. Concentrations of phytoplankton <10 µm for all tests. These organisms are not part of D-2. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power. Efficacy is the logarithmic reduction of viable organisms in treated water on discharge (T5) compared to the total phytoplankton concentration in the test water.

set I						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	18,910	35,581	8,028	31,932	278	2.1
2	18,910	35,581	8,028	32,935	176	2.3
3	10,779	13,552	1,067	22,242	9	3.1
4	10,779	13,552	1,067	11,802	15	2.9
5	699	3,108	733	3,023	3	2.9
6	1,174	3,258	701	6,139	0	3.5
average	10,209	17,439	3,271	18,012	80	2.8

set II						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	2,766	3,638	238	5,569	6	2.7
2	2,766	3,638	238	4,565	4	2.9
3	699	3,108	733	3,454	3	2.9
4	1,174	3,258	701	1,980	0	3.5
5	2,466	4,900	873	6,564	6	2.8
6	2,466	4,900	873	10,211	2	3.2
average	2,056	3,907	609	5,391	4	3.0

set III						
		Control		Treated		
Test	Test water	T0	T5	T0	T5	Efficacy
1	15,982	16,828	352	9049	20	2.9
2	15,982	16,828	352	8856	21	2.9
3	4,715	4,313	556	3505	44	2.0
4	4,715	4,313	556	2142	10	2.6
5	1,684	1,928	184	3510	*	*
average	8,616	8,842	400	5,412	24	2.6

*not measured

The small phytoplankton was a factor 2 to 10 more abundant in the in the test water and in the control at T0 than the larger 10≤µm<50 phytoplankton (Table 5.6). In general, the concentrations of viable <10 µm phytoplankton cells was less than 100 per mL. In test 4 that failed on a slightly too high >50 µm organism concentration due to a leaking seal in the filtration unit no viable 10≤µm<50 and <10 µm cells were detected although a small peak in the PAM fluorimeter had been detected.

The efficacies that were obtained for the <10 µm phytoplankton, ranging from 2.6 to 3.0, were comparable to those of the 10≤µm<50 phytoplankton (Table 5.11).

The Aquarius™-UV system performed very well in reducing the cell concentrations of <10 µm phytoplankton, for which the D-2-standard does not apply, with efficacies comparable to those for 10≤µm<50 phytoplankton.

5.4.3 Incubation experiments

Incubation experiments were performed to assess the viability of phytoplankton and microzooplankton upon discharge. Organism cells may be intact after the second UV-treatment but are probably not vital anymore, i.e. dead and not able to reproduce. To measure the ability of phytoplankton and microzooplankton to reproduce, which is their viability, 10 L discharge samples were incubated under optimal conditions.

Table 5.12. Viability as average growth rates (divisions per day) of two size classes of phytoplankton calculated from total phytoplankton concentrations on day zero and day seven of incubation experiments. The rates are ±95% confidence intervals. Negative rates indicate loss of cells. Set I and II were performed at the intermediate salinity range and at 60% and 100% UV power respectively. Set III was performed at the high salinity range at 100% UV power.

	Phytoplankton	
Set	10≤µm<50	<10 µm
I	0.1 ± 0.5	-1.0 ± 0.6
II	0.0 ± 0.3	-0.4 ± 0.3
III	-0.4 ± 0.6	-1.0 ± 0.5

Incubation of the Aquarius™-UV treated water under favourable conditions for phytoplankton growth showed that after a seven day period all tests showed that either no growth occurred, the averages were not significantly different from zero, or that cell concentrations declined. A significant decline in 10≤µm<50 sized phytoplankton occurred in all treatments (Table 5.12) although the averages per treatment were not significantly different. Apparently, the presence of vital cells in discharge water, as measured with SYTOX-Green on T5, does not necessarily mean that these cells are also viable.

In 2012 the viability of the microzooplankton (10≤µm<50) was measured in the same incubation experiments that were performed for the phytoplankton. However, because the microzooplankton concentrations in this year were usually very small (<0.3 per mL), accurate enumerations were difficult to make. Therefore, in only two treatments, one at 60% and one at 100% UV-power, with relatively high microzooplankton concentrations, daily measurements of the concentrations of intact microzooplankton in the incubation experiments were made. These experiments showed that in control, untreated samples, the microzooplankton concentrations increased while in the treated (discharge) water their abundance declined to undetectable concentrations after only one day of incubation (Figure 4.3). The conclusion from these microzooplankton incubations was that morphologically intact organisms in discharge water cannot *a priori* be considered viable.

The incubation experiments showed that morphologically intact or apparently vital organisms in treated discharge water may not be viable. In other words, vitality measurements may overestimate the viability of organisms.

5.5 Summary statistical analysis

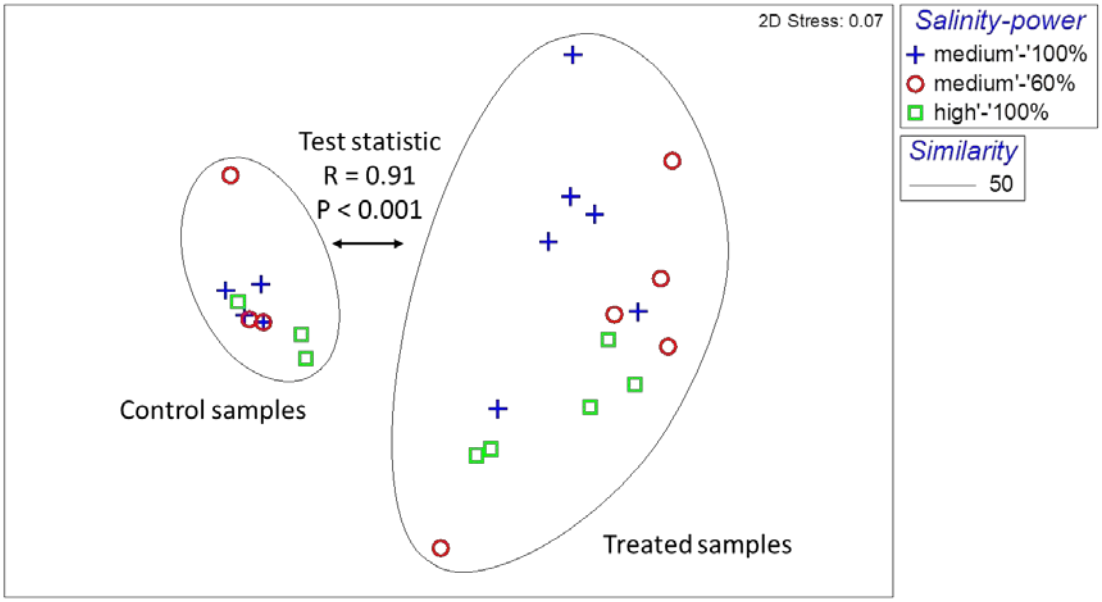

Figure 5.2. Diagram of the mathematical distances between control and treated samples at T5 calculated from total phytoplankton, microzooplankton and mesozooplankton concentrations. This diagram does not have numerical axes.

The overall effect of the ballast water treatment is presented in Figure 5.2. This statistical analysis was based on the concentrations of total phytoplankton (10≤µm<50) and microzooplankton (10≤µm<50) and viable mesozooplankton (>50 µm). The control samples cluster together because they are over 50% similar. The UV-treated samples are also over 50% similar to each other. However, the control and filter+UV-treated samples differ significantly from each other. The test statistic R from a non-parametric ANOVA is very high (R = 0.91). The chance P that this difference occurs by chance is less than 1 in 1000 (P<0.001). Therefore, the null-hypothesis that treated and control samples are equal is rejected: there is a very significant effect of the treatment compared to the controls.

The control samples are significantly different from the samples treated with the Aquarius™-UV BWTS.

6 CONCLUSIONS

Differences between all control and treatment tests

- There was no statistical significant overall effect of the Aquarius™-UV ballast water treatment on abiotic variables (temperature, pH, oxygen saturation, TSS, POC, DOC).
- A statistical test between all control samples and all samples treated by the Aquarius™-UV system indicated a highly significant overall effect on the biotic variables (total phytoplankton (10≤µm<50), microzooplankton (10≤µm<50) and viable mesozooplankton (>50 µm)).

G8-requirements

For the G8-requirements regarding the abiotic and the biological test water quality it is concluded that:

- The abiotic quality of the naturally available test water met the requirements for conclusive testing of the Aquarius™-UV system.
- The requirements for biological quality of the test water during the test of the Aquarius™-UV system were met. A shortage of numbers in the high salinity tests was compensated by a high biodiversity in all tests.
- The concentrations of heterotrophic bacteria were well above the G8-guideline for test water at intake.
- Treatment with the Aquarius™-UV system reduced TSS and POC concentrations. Oxygen saturation levels were lowered as well but remained high enough to prevent local hypoxic conditions.

D-2-requirements

In relation to the Ballast Water Performance Standard (D-2) it is concluded that:

- The data from the testing of the Aquarius™-UV system strongly demonstrates that following the second UV treatment the concentration of organisms ≥50µm meets the level stipulated in D-2 standard. These test results clearly demonstrate the efficacy of the treatment to deliver compliant results.
- The data from the testing of the Aquarius™-UV system strongly demonstrates that following the second UV treatment the concentration of all 10≤µm<50 organisms, i.e. the sum of the viable phytoplankton and microzooplankton concentrations, meets and exceeds the level stipulated in D-2 standard. These tests demonstrate the high level of treatment efficacy of the Aquarius™-UV system.
- All samples tested for *E. coli* and for enterococci fulfilled the D-2-standard.
- These conclusions are valid for the tests with 100% and 60% UV-power.

Calculated efficacies

- The biological efficacies at all UV-powers tested surpassed the combined D2-G8 requirement of 2.0 (10≤µm<50 organisms) and 4.0 (>50 µm organisms) with values of 2.6 to 3.3 and 4.5 to 4.6. These efficacies indicate a 2,000x (10≤µm<50 organisms) to 30,000x (>50 µm organisms) reduction where 100x and 10,000x are required.

Final conclusion

The configuration of the Aquarius™-UV system as tested at NIOZ in 2011 and 2012 is an environmentally safe ballast water treatment system with a high efficacy that meets and exceeds the reductions of viable organisms in the required size classes as stipulated in the D-2 Ballast Water Performance Standard.

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Appendix 1. List of species/species groups observed in the spring/summer testing season of 2011 and 2012.

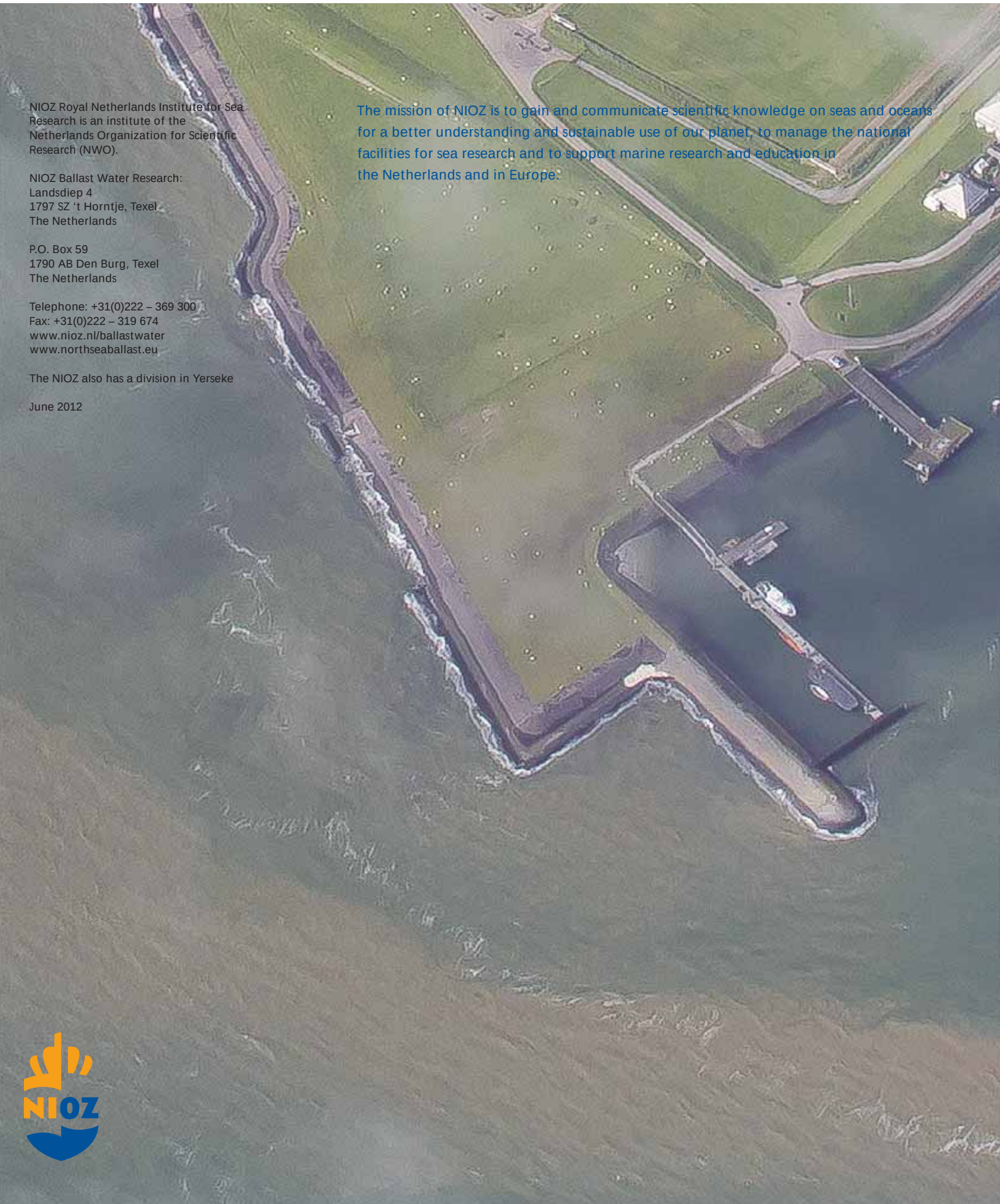
Species/species group name	phylum	<10 µm	10-50 µm	>50 µm
Acarti clausi	Arthropoda			1
Actinoptychus senarius	Ochrophyta		1	
Akashiwo sanguinea	Myzozoa		1	
Apedinella spinifera	Ochrophyta	1		
Askenasia	Ciliophora	1		
Asterionella formosa	Ochrophyta	1		
Asterionellopsis glacialis	Ochrophyta	1		
Asteroplanus karianus	Ochrophyta	1		
Bacillariales_indet_<10u	Ochrophyta	1		
Bacillariales_indet_>30u	Ochrophyta		1	
Bacillariales_indet_10-30u	Ochrophyta		1	
Balanid Cypris and nauplia	Arthropoda			1
Balanion	Ciliophora	1		
Bivalvia_larva_	Mollusca			1
Brockmanniella_brockmannii_<10u	Ochrophyta	1		
Bryozoa	Bryozoa			1
Centropages spec.	Arthropoda			1
Ceratium fusus	Myzozoa		1	
Ceratoneis cf. closterium	Ochrophyta	1		
Chaetoceros simplex	Ochrophyta	1		
Chaetoceros_spp_<10u_colony	Ochrophyta	1		
Chaetoceros_spp_<10u_solitar	Ochrophyta	1		
Choanoflagellatea	Choanozoa	1		
CHOREOTRICHIDA	Ciliophora		1	
Chromobiota < 03 µm	Chromista	1		
Chromobiota 03-10 µm	Chromista	1		
Chromobiota sp. 1	Chromista	1		
Chrysochromulina	Haptophyta	1		
Clytia gracilis	Cnidara			1
Copepoda	Arthropoda			1
Coscinodiscus radiatus	Ochrophyta			1
Crustacea_larva	Arthropoda			1
Cryptomonadales > 10 µm	Cryptophyta		1	
Ctenophora	Ctenophora			1
Cyanophyta_indet_(cell=1)	Cyanobacteria	1		
CYCLOTRICHIDA	Ciliophora			1
Delphineis minutissima	Ochrophyta	1		
Dinophyta_indet_colored_>30u	Myzozoa		1	
Ditylum brightwellii	Ochrophyta		1	
Ebria tripartita	Protozoa incertae sedis		1	
ECHINODERMATA	Echinodermata			1
Eunotogramma_dubium	Ochrophyta		1	
Euplotes	Ciliophora		1	
Eutreptiella	Euglenozoa		1	
flagellate_indet_colored_~3u	unknown	1		
flagellate_indet_colored_<10u	unknown	1		
flagellate_indet_colored_10-30u	unknown		1	
flagellate_indet_colorless_<6u	unknown	1		
flagellate_indet_colorless_6-10u	unknown	1		

Appendix 1 (continued). List of species/species groups observed in the spring/summer testing season of 2011 and 2012.

Species/species group name	phylum	<10 µm	10-50 µm	>50 µm
Fragilariaceae_indet	Ochrophyta	1		
Gastropoda	Mollusca			1
Guinardia_delicatula	Ochrophyta		1	
Guinardia_flaccida	Ochrophyta			1
Gymnodiniaceae_>30u	Myzozoa		1	
Gymnodiniaceae_10-30u	Myzozoa	1		
Gyrodinium_spirale_group	Myzozoa		1	
Gyrosigma fasciola	Ochrophyta		1	
Haptoria	Ciliophora		1	
HAPTORIDA	Ciliophora		1	1
Harpacticoida	Arthropoda			1
Hemiselmis_group_2-9u	Cryptophyta	1		
HYMENOSTOMATIA	Ciliophora	1	1	1
Hypotrichia	Ciliophora		1	
Katodinium glaucum	Myzozoa		1	
Khakista < 10 µm	Ochrophyta	1		
Khakista < 10 µm b. < 50 µm l.	Ochrophyta		1	
Kolkwitziellaceae	Myzozoa		1	
Laboea strobila	Ciliophora			1
Lanice	Annelida			1
Leegaardiella sp	Ciliophora		1	
Licmophora	Ochrophyta		1	
Lithodesmium_undulatum	Ochrophyta			1
Lohmanniella oviformis	Ciliophora		1	
Mediopyxis_helysia	Ochrophyta		1	
Micromonas pusilla	Chlorophyta	1		
Myrionecta rubra	Ciliophora		1	
Nematoda	Nematoda			1
Nephtid/Nereid larvae	Annelida			1
Nitzschia	Ochrophyta		1	
Noctiluca_scintillans_group	Myzozoa			1
Odontella_aurita	Ochrophyta			1
Odontella_regia	Ochrophyta			1
Odontella_rhombus	Ochrophyta			1
Odontella_sinensis	Ochrophyta			1
Oithona similis	Arthropoda			1
OLIGOTRICHIDA	Ciliophora		1	
Paralia_sulcata	Ochrophyta	1		
Paulinella_group	Cercozoa		1	
Peridiniales 10-30 µm	Myzozoa		1	
Peridiniales 30-50 µm	Myzozoa		1	
PERITRICHIA	Ciliophora		1	
Phaeocystis_globosa_colony_cell	Haptophyta	1		
Phaeocystis_globosa_flagellate_cell	Haptophyta	1		
Phyllodocidae (larvae and little)	Annelida			1
Plagiogrammopsis_vanheurckii	Ochrophyta	1		
Plagioselmis_group_5-11u	Ochrophyta	1		
Polychaeta larvae (late)	Annelida			1
Polycheles	Arthropoda		1	
Prasinophyceae_Pseudocourfieldia_group_~3u	Chlorophyta	1		

Appendix 1 (continued). List of species/species groups observed in the spring/summer testing season of 2011 and 2012.

Species/species group name	phylum	<10 µm	10-50 µm	>50 µm
Protomonadales	unknown	1		
Protoperidinium achromaticum	Myzozoa			1
Protoperidinium pellucidum	Myzozoa		1	
Protoperidinium_bipes	Myzozoa		1	
Prymnesiales_6-10u	Haptophyta	1		
Pseudocalanus spec.	Arthropoda			1
Pseudochattonella	Ochrophyta		1	
Pseudo-nitzschia_delicatissima_group	Ochrophyta	1		
Pseudo-nitzschia_pungens_group	Ochrophyta	1		
Pterospermataceae	Chlorophyta	1		
Pyramimonas < 10 µm	Chlorophyta	1		
Raphidophyceae	Ochrophyta		1	
Rhizosolenia_imbricata_group	Ochrophyta		1	
Rimostrombidium	Ciliophora			1
Scrippsiella	Myzozoa		1	
Skeletonema cf. costatum	Ochrophyta	1		
Skeletonema_lenses	Ochrophyta		1	
Spionida (larvae)	Annelida			1
Strobilidium	Ciliophora		1	1
Strombidium	Ciliophora		1	1
Strombidium acuminatum	Ciliophora			1
Strombidium minor	Ciliophora		1	
Strombidium sp 'parachute'	Ciliophora			1
Teleaulax_acuta_group_12-18u	Ochrophyta	1		
Temora longicornis	Arthropoda			1
Thalassionema_nitzschioides	Ochrophyta	1		
Thalassiosira 30-80 µm	Ochrophyta		1	
Thalassiosiraceae_indet_<6u	Ochrophyta	1		
Thalassiosiraceae_indet_>30u	Ochrophyta		1	
Thalassiosiraceae_indet_10-30u	Ochrophyta		1	
Tintinnida (groep 1a)	Ciliophora	1	1	
Tintinnidium balechi	Ciliophora		1	
Tintinnopsis	Ciliophora	1	1	
Tintinnopsis beroidea	Ciliophora	1	1	
Tintinnopsis nana cf	Ciliophora	1		
Tintinnopsis parvula	Ciliophora		1	
Torodinium robustum	Myzozoa		1	
Tryblionella_coarctata_group	Ochrophyta		1	
Vorticella	Ciliophora		1	
Warnowia	Myzozoa		1	
Zoea larvae	Arthropoda			1



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The NIOZ also has a division in Yerseke

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